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HYDROLYTIC STABILITY OF FLAVOR ENHANCING 5'-PURINE RIBONUCLEOTIDES

by

Orly Shaoul

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Fall 1986

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled **HYDROLYTIC STABILITY OF FLAVOR ENHANCING 5'-PURINE RIBONUCLEOTIDES** submitted by **Orly Shaoul** in partial fulfilment of the requirements for the degree of **Master of Science** in **Food Chemistry.**

.....Peter Lasko.....

Supervisor

.....

.....

Date. June 16, 1986

To Mark for his love, support and

encouragement throughout my M.Sc. program

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ABSTRACT

Two commonly used food additives are guanosine 5'-monophosphate (GMP) and inosine 5'-monophosphate (IMP). Adenosine 5'-monophosphate (AMP) is a nucleotide which is ordinarily found in animal tissues. These 5'-purine nucleotides are very labile under canning conditions (121°C) and hydrolyse to their corresponding nucleosides and bases. This was detected using High Performance Liquid Chromatography (HPLC).

A detailed analysis was performed to study the influence of pH during processing in order to understand 5'-nucleotide hydrolysis in foods. It was found that the nucleotides degrade via a first-order reaction and the maximum rate of hydrolysis between pH 4-8 occurred at pH 5. Two mechanisms, phosphate and base hydrolyses, were observed at pH 3, whereas phosphate hydrolysis was predominant at pH 4-8, inclusive. Accelerated shelf life testing using the Arrhenius method revealed that the half lives of IMP, GMP and AMP were 35.68, 18.65 and 39.66 years, respectively, at 23°C and pH 5; thus these compounds are very stable at room temperature.

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1. INTRODUCTION

1.1 5'-Purine Ribonucleotides

1.1.1 History

In 1847 Liebig was the first to isolate a nucleotide, IMP from meat (Kuninaka, 1981). In 1913, Dr. Kodama of Tokyo University discovered the flavor enhancing properties of 5'-nucleotides (Sjöström, 1972). Dr. Kodama had been searching for the ingredient in bonito tuna which made it effective as a flavor enhancer. His conclusion was that the ingredient was inosine 5'-monophosphate (IMP). It was not produced commercially and employed as a flavor enhancer until 50 years later (de Man, 1980) when the relationship between chemical structure and flavor activity of the nucleotide was discovered and a process for preparing the compound from ribonucleic acid (RNA) was developed (Kuninaka, 1967). Later, establishment of the RNA degradation process led to the recognition of the flavor enhancing properties of guanosine 5'-monophosphate (GMP) (Kuninaka *et al.*, 1964).

1.1.2 Distribution of 5'-purine ribonucleotides

The water soluble components of foods cause the sensations of taste and are the components which contribute to the flavor of foods. Nucleotides, amino acids, peptides, organic acids, sugars, organic bases such as creatine,

betaines, etc., and inorganic ions are included among the water soluble components in foods. Each component has at least one of the tastes identified as saltiness, sweetness, sourness, bitterness or savoriness. Thus, the concentration of the components, the interactions among them and pH change the taste quality accordingly. Among the principal taste components in both animal and vegetable foods are nucleotides such as inosine 5'-monophosphate, guanosine 5'-monophosphate and adenosine 5'-monophosphate (AMP) (Ajinomoto Tech. Bull., 1982a).

Tables 1.1 and 1.2 show the nucleotide content in natural foods. In 1982 Motono observed the following:

(1) Animal foods, such as beef, pork, chicken and dried bonito contain a great amount of IMP.

(2) Crustacea and mollusca, like squid, lobster and abalone, and almost all vegetables contain AMP.

(3) The dried mushroom "Shiitake" contains a great amount of GMP and in Japan and China it is the traditional ingredient used in producing soup stock.

1.1.3 Relationship between taste and chemical structure of nucleotides

The commercial use of 5'-nucleotides was delayed so long because it was not clear which of three isomers of IMP had the flavor enhancing properties. To solve the problem, three isomers were prepared and their flavor effects were checked. 2'-IMP and 3'-IMP were prepared from RNA and 5'-IMP

Table 1.1 Distribution of nucleotides in animal foods.

Food	Nucleotide content (mg/100 g) IMP	GMP	AMP	Reference
Beef	163	---	7.5	a
Pork	186	3.7	8.6	a
Chicken	115	2.2	13.1	a
Whale	326	5.3	2.4	a
Horse mackerel	323	0	7.2	a
Sweet fish	287	0	8.1	a
Common sea bass	188	0	9.5	a
Pilchard	287	0	0.8	a
Black sea bream	421	0	12.4	a
Pike mackerel	227	0	7.6	a
Mackerel	286	0	6.4	a
Keta salmon	235	0	7.8	a
Tuna	286	0	5.9	a
Globefish	287	0	6.3	a
Eel	165	0	20.1	a
Dried bonito	630-1310	0	trace	a
Squid	0	0	184	a
Common octopus	0	0	26	a
Spiny lobster	0	0	82	a
Hairy crab	0	0	11	a
Squilla	26	0	37	a
Common abalone	0	0	81	a
Round clam	0	0	98	a
Common scallop	0	0	116	a
Shortneck clam	0	0	12	a
Sardine	192.6	---	6.6	b
Perch	124.9	0	8.4	b
Codfish	43.8	---	23.9	b
Swordfish	19.9	0	3.1	b
Rainbow trout	117	---	14.6	b
Trout	187	---	4.2	b
Oyster	0	0	21	b
Prawn (shrimp)	---	---	11.5	b

a -- Ajinomoto Tech. Bull., 1982b.

b -- Considine, 1982.

Table 1.2 Distribution of nucleotides in vegetable foods.

Food	Nucleotide content (mg/100 g) IMP	GMP	AMP	Reference
Asparagus	0	trace	4	a
Welsh onion	0	0	1	a
Head lettuce	trace	trace	1	a
Tomato	0	0	12	a
Green pea	0	0	2	a
Cucumber	0	0	2	a
Japanese radish	trace	0	2	a
Onion	trace	0	1	a
Bamboo shoot	0	0	1	a
Mushroom:				
"Bentitengu dake"	0	0	trace	a
"Naratake"	0	0	trace	a
"Shiitake"	0	103	175	b
"Shiitake", dried	0	216	321	b
French	0	trace	13	b
French, dried	0	trace	190	b
"Enokidake"	0	32	45	b
"Matsutake"	0	95	112	b
"Syorô"	0	9	16	b
"Hatsutake"	0	85	58	b
Corn	0	0	6.5	c
Green beans	---	---	1.8	c

a -- Ajinomoto Tech. Bull., 1982b.

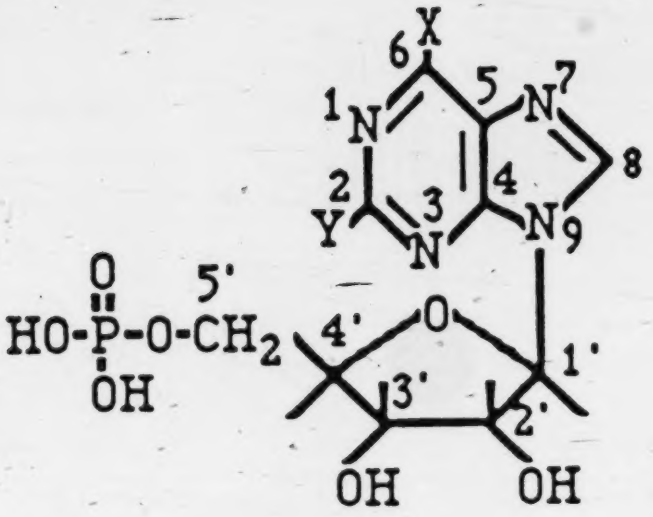
b -- Motono, 1982.

c -- Considine, 1982.

was isolated from animal muscle tissue. Among the three isomers, only 5'-IMP had flavor activity (Kuninaka, 1967).

Purine and pyrimidine bases, nucleosides and polynucleotides have little recognizable taste, whereas mononucleotides have a pleasant taste. (A nucleotide consists of a nucleoside moiety and a phosphate moiety, and the nucleoside moiety is further subdivided into a sugar moiety and a base moiety.) The taste of 5'-nucleotides is much stronger than that of 2'- or 3'-nucleotides (Kuninaka, 1964).

The chemical structure of a nucleotide (Figure 1.1), in which a purine nucleus has a hydroxyl group at the 6-position and the 5'-position of the ribose moiety is esterified by phosphoric acid, is required for the flavoring action (Yamaguchi *et al.*, 1971). Both hydroxyls on the phosphate group are essential and both primary and secondary dissociations are also necessary for flavor activity (Kuninaka, 1964). Since the intensity of the flavoring activities of compounds related to 6-hydroxypurine ribonucleoside 5'-phosphate, such as IMP and GMP, differ considerably from one another, it has been proposed that the group at the 2-position would affect the magnitude of the taste intensity (Yamaguchi *et al.*, 1971). The flavor activity of GMP is greater than that of IMP. Replacement of the 6-hydroxyl group in the flavor nucleotide by an amino group results in the reduction of the flavor activity. Therefore, AMP is less active than GMP or IMP (Kuninaka,



	IMP	GMP	AMP
X=	OH	OH	NH_2
Y=	H	NH_2	H

Figure 1.1 Chemical structure of nucleotides.

1981).

The 2'- and 3'-hydroxyl groups in IMP or GMP are not essential because the flavor activity can be detected in 5'-deoxyinosinate and 5'-deoxyguanylate, since the 2'-hydroxyl group can be replaced by a hydrogen without total loss of flavor activity (Peterson and Johnson, 1978). It has also been determined that the hydrogen atoms of the 2'- and 3'-hydroxyl groups are not necessarily essential for flavor activity, although the principal part of the ribose molecule is essential (Kuninaka, 1966). This was determined since flavor activity was reported in inosine 2'(3'),5'-diphosphate and guanosine 2'(3'),5'-diphosphate.

1.1.4 Industrial production of 5'-purine ribonucleotides

In 1959 a Japanese firm began commercial production of 5'-IMP and 5'-GMP (Sjöström, 1972). In 1962 the Food and Drug Administration (USA) approved them as food additives and in 1963 an American firm began marketing them in the United States (Peterson and Johnson, 1978).

There are several methods for producing 5'-nucleotides: (1) *Enzymatic degradation of RNA*. RNA is a polynucleotide in which individual nucleoside residues are joined, one to the other, by phosphodiester linkages between the 3'- and 5'-positions. When RNA is degraded, cleavage must be considered to occur at either the 3'-phosphodiester linkage (C_3'-O†P(O_2H)-O-C_5') or the 5'-phosphodiester linkage (C_3'-O-P(O_2H)†O-C_5'). The latter cleavage results in

nucleotides without flavor activity (Kuninaka *et al.*, 1964), that is, no 5'-phosphate group.

Enzymes in *Penicillium* strains and *Streptomyces* strains, from which the 5'-phosphodiesterase type can be obtained in large amounts, are used in the industrial production of 5'-nucleotides from yeast RNA (Kuninaka, 1966).

(2) *Combination of nucleoside fermentation and chemical phosphorylation.* Fermentative production of nucleosides is relatively easy, especially for inosine. Inosine can then be chemically phosphorylated to 5'-inosinic acid (Kuninaka, 1966), as shown in Figure 1.2.

(3) *Direct nucleotide fermentation.* It has been shown that 850 mg of 5'-AMP per l were accumulated by a wild strain of microorganisms (Kuninaka *et al.*, 1964). It is more difficult to accumulate large amounts of 5'-nucleotides using microorganisms than to accumulate the same amounts of nucleosides.

(4) *Chemical decomposition of RNA into nucleosides and their chemical phosphorylation* (Kuninaka, 1981).

1.1.5 Safety of 5'-purine ribonucleotides

According to the FAO/WHO, the establishment of an acceptable daily intake of IMP and GMP is not deemed necessary (Kuninaka, 1981). The total daily intake of the nucleotide arising from its use at the levels necessary to achieve the desired effect, coupled with its acceptable background in foods, does not represent a hazard to health.

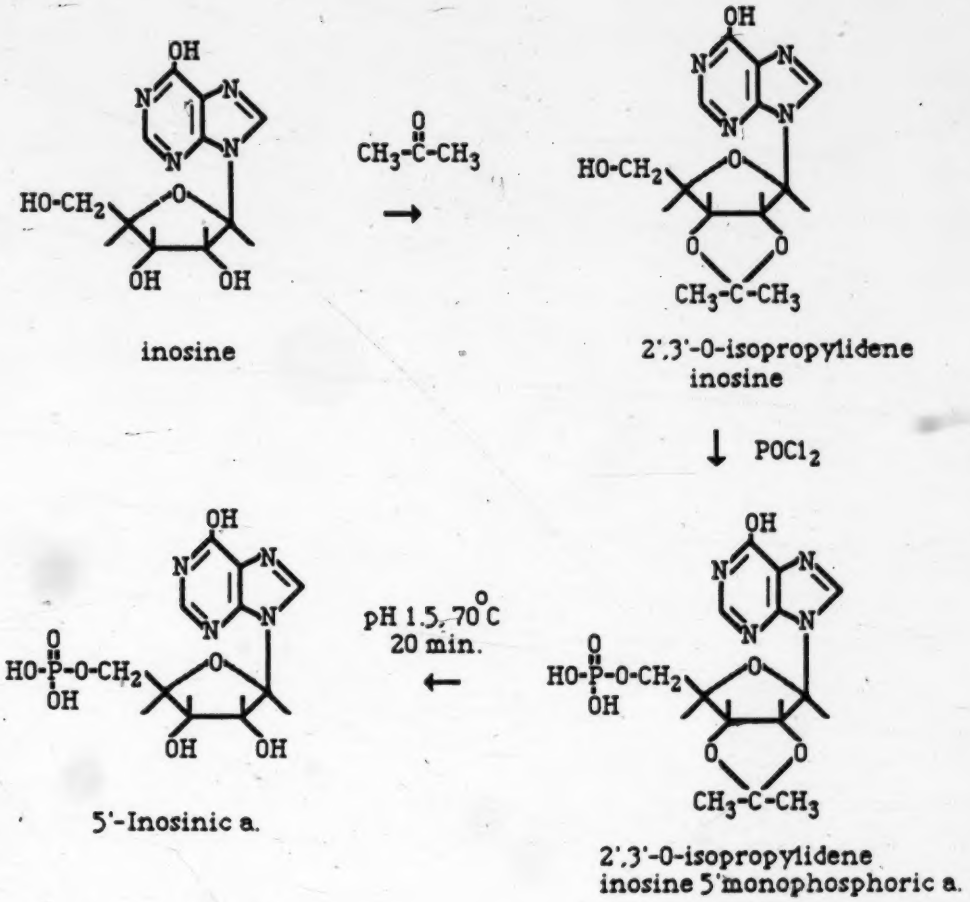


Figure 1.2 Flow diagram for the production of inosine 5'-monophosphate. Source: Kuninaka (1966).

1.2 Heat Preservation

1.2.1 Basic food processing principles

The use of heat to preserve foods is based on the principles of:

(1) destruction of pathogens,

(2) destruction of spoilage microbes,

(3) denaturation of enzymes, and

(4) softening of tissues to make them more digestible (Labuza, 1982).

1.2.2 Sterilization

The objective of the sterilization process is to destroy the microorganisms present in the food and the container that might cause spoilage of the canned product under normal handling and storage conditions. Canned foods are "commercially sterile", but not bacteriologically sterile since the sterilization process is not designed to kill all microorganisms in canned foods (Lopez, 1969). Processing to destroy all organisms will destroy the food quality. If adequate heat treatment is used, canned "commercially sterile" foods lose some color, lose 20-30% of some vitamins, have a cooked flavor and develop a softer texture. Subsequent modes of deterioration are confined to chemical modes of decay which do not require oxygen since the can is hermetically sealed (Labuza, 1982).

The thermal conditions required to produce commercial sterility depend on many factors, including:

(1) nature of food (e.g. pH),

(2) storage conditions of the food following the thermal process,

(3) heat resistance of the microorganisms or spores,

(4) heat-transfer characteristics of the food, its container and the heating medium, and

(5) initial load of microorganisms (Lund, 1975).

For thermal process design, foods are divided into three pH groups:

(1) high-acid foods with pH values less than 3.7,

(2) acid foods with pH values from 3.7 to 4.5, and

(3) low-acid foods with pH values greater than pH 4.5.

Tables 1.3 and 1.4 contain examples of foods in the high acid/acid and low-acid groups, along with their pH values (Lund, 1975).

The main concern of the canning industry in canned food sterilization is to prevent the growth of *Clostridium botulinum*, a food poisoning bacterium capable of producing a highly lethal toxin (Lopez, 1969).

A sterilization process that assures the destruction of *Cl. botulinum* also kills all other microorganisms capable of producing canned food spoilage under normal conditions of canned food handling and storage since *Cl. botulinum* is a highly heat resistant bacterium (Lopez, 1969).

A study was made of the growth necessities for *Cl.*

Table 1.3 Canned foods with pH values less than 4.5 (acid foods).

Canned product	Average pH value
Apples	3.4
Apple sauce	3.6
Apricots	3.9
Blackberries	3.5
Blueberries	3.4
Cherries:	
black	4.0
red sour	3.5
Royal Ann	3.8
Cranberry sauce	2.6
Grape juice	3.2
Grapefruit juice	3.2
Lemon juice	2.4
Loganberries	2.9
Orange juice	3.7
Peaches	3.8
Pears, Bartlett	4.1
Pickles:	
fresh cucumber	3.9
sour dill	3.1
sweet	2.7
Pineapple juice	3.5
Plums:	
Green Gage	3.8
Victoria	3.0
Prunes, fresh purine plums	3.7
Raspberries:	
black	3.7
red	3.1
Sauerkraut	3.5
Strawberries	3.4
Tomatoes	4.3
Tomato juice	4.3
Tomato purée	4.4

Source: Lund (1975).

Table 1.4 Canned foods with pH values greater than 4.5 (low-acid foods).

Canned product	Average pH value
Asparagus:	
green	5.5
white	5.5
Beans:	
baked	5.9
green	5.4
lima	6.2
wax	5.3
Beans and pork	5.6
Beets	5.4
Carrots	5.2
Corn:	
whole grain, brine packed	6.3
cream style	6.1
Figs	5.0
Mushrooms	5.8
Olives, ripe	6.9
Peas:	
Alaska	6.2
sweet, wrinkled	6.2
Potatoes:	
sweet	5.2
white	5.5
Pumpkin	5.1
Spinach	5.4

Source: Lund (1975).

botulinum and it was found that the dividing line of acidity between products in which the organism would grow and those in which it would not grow was about pH 4.5. Products with pH levels greater than 4.5 must be processed under pressure at temperatures above 121°C in order to ensure destruction of spores, whereas products at pH 4.5 or lower may be safely processed at 121°C (Lopez, 1969).

Cl. botulinum is assumed to be present on all products intended for canning since it is widely distributed in nature. The most heat resistant strains of *Cl. botulinum* which produce toxin are types A and B. The toxin is extremely potent (one millionth of a gram will kill a man) but can be destroyed when exposed to heat for ten minutes at 121°C (Lund, 1975).

1.2.3 Interaction of heat energy and food components

1.2.3.1 Reaction kinetics

A majority of reactions that occur in foods obey well-established kinetics. The thermal destruction of micro-organisms, most nutrients, quality factors (texture, color, flavor) and enzymes generally obey first-order reaction kinetics, hence, the destruction rate of each of these components is dependent on the concentration of the component (Lund, 1975).

Microorganism are the basis of thermal process calculations, therefore microbial inactivation will be used to illustrate first-order kinetics. Expressing the first

order response gives:

$$-dc/dt = kc \quad (1)$$

where

-dc/dt is the rate at which concentration decreases

c is the concentration of the viable microorganisms

k is the first-order reaction rate constant

Integrating between limits, c_1 at time t_1=0 and c at time t, results in:

$$-\int_{c_1}^{c} dc/c = k\int_{t_1}^{t} dt \quad (2)$$

$$-\ln c + \ln c_1 = k(t-t_1) \quad (3)$$

$$\log c = \log c_1 - kt/2.303 \quad (4)$$

Figure 1.3 is a graphic expression of the equation. The number of survivors for any heating time can be obtained directly from the curve. The slope of the line is -k/2.303. The time required for the survivor curve to transverse one log cycle corresponds to a 90% reduction in the number of survivors. Therefore, the "decimal reduction time" or D value is the time required to reduce the population by 90% and the slope of the survivor curve is expressed as:

$$(-\log c_1 - \log c)/D = (-\log c_1/c)/D$$

$$= (-\log 100/10)/D = -1/D \quad (5)$$

Since the slope is also -k/2.303,

$$-k/2.303 = -1/D \quad or \quad D = 2.303/k \quad (6)$$

(Lund, 1975).

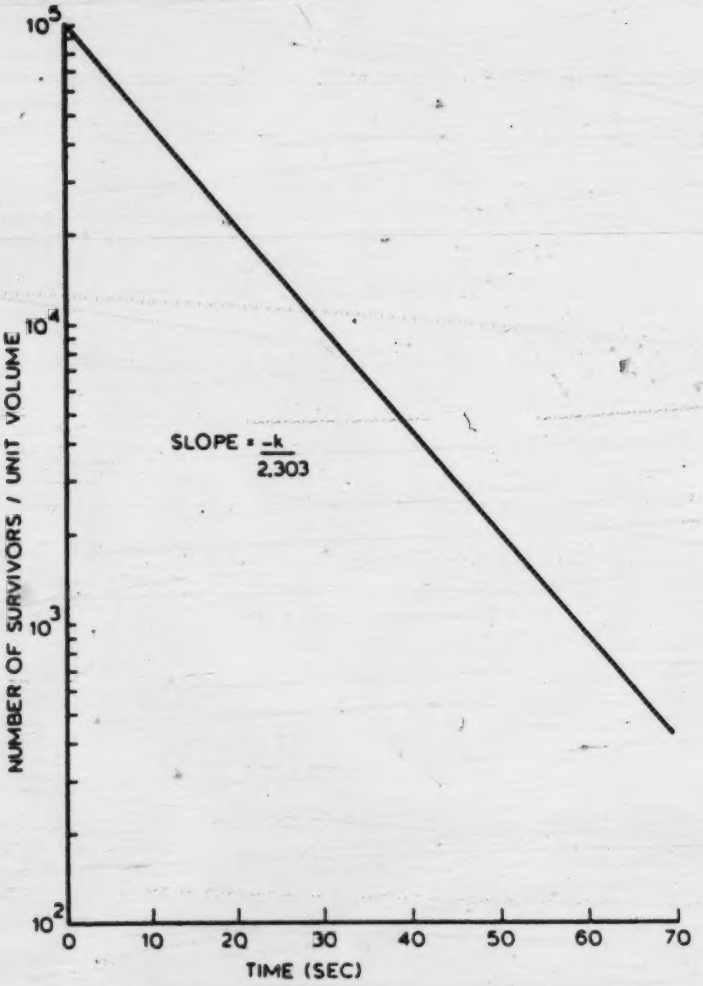


Figure 1.3 First-order destruction of microorganisms. Source: Lund (1975).

1.2.3.2 Temperature dependence of kinetics

Real food products do not heat instantaneously to 121°C but go through a time-dependent temperature treatment. Therefore, the rate of inactivation at several temperatures must be known. There are two main methods of describing the dependence of the reaction rate constant on temperature:

(1) the Arrhenius equation, and

(2) thermal death time curves (Lund, 1975).

The dependence of the reaction rate constant on temperature is described as the Arrhenius equation:

$$k = k_0 \exp(-E_a/RT) \quad (7)$$

where

k is the reaction rate constant (min^{-1})

k_0 is a constant, the frequency factor (min^{-1})

E_a is the activation energy (cal/mole)

R is the gas constant (1.987 cal/mole-°K)

$\overline{T}$ is the absolute temperature (°K)

Taking the logarithms of both sides results in:

$$\ln k = \ln k_0 - E_a/RT \quad (8)$$

Thus, a plot of ln k versus the reciprocal of the absolute temperature is a straight line (Lund, 1975). Three systems are theoretically represented in Figure 1.4. The slope of each line is equal to the activation energy divided by the gas constant. A steeper slope suggests the reaction is more temperature dependent, that is, as the temperature increases, the reaction increases at a more rapid rate. Therefore, in Figure 1.4, B and C have the same temperature

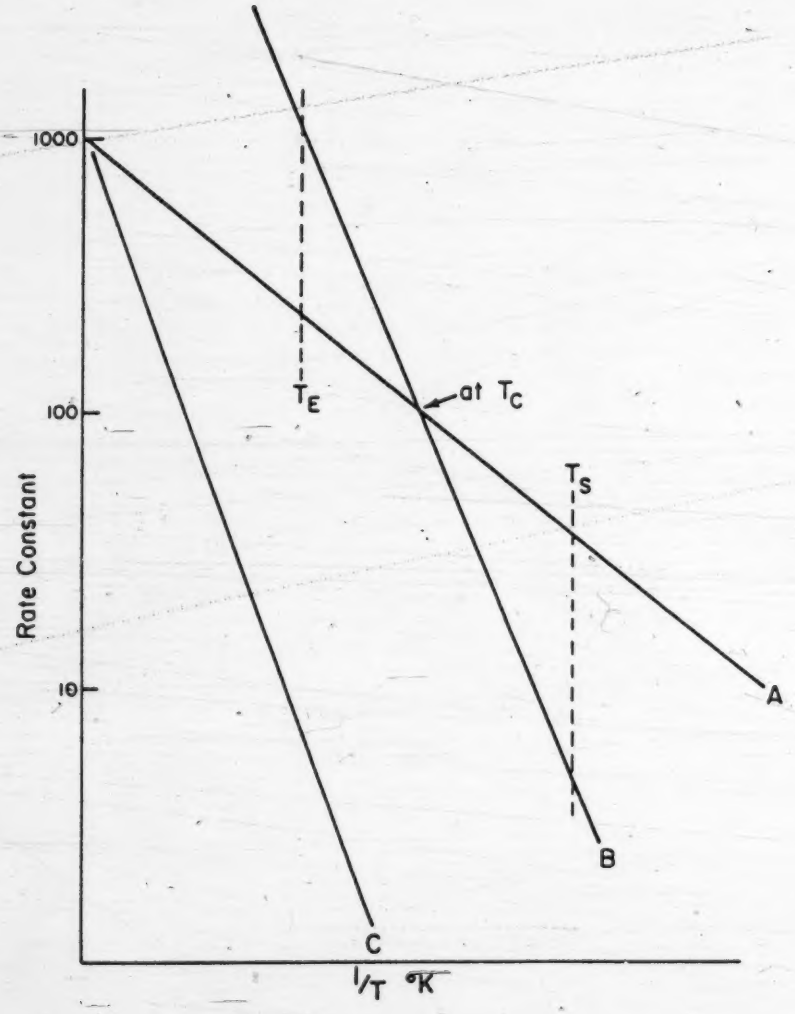


Figure 1.4 Arrhenius plots. Source: Labuza (1982).

dependence, and increase in rate faster than A as temperature increases. Also, at some possible temperature, the rates are the same, but above and below this level their relative rates are different. At temperatures below T_c, such as at T_s, the rate of loss of A is faster than B; above T_c, such as at T_E, B is lost more rapidly than A (Labuza, 1982).

The frequency factor k_0 can be evaluated by allowing the reaction rate constant to be k_1 at temperature T_1. Then:

$$\ln k_0 = \ln k_1 + E_a/RT_1 \qquad (9)$$

Substitution of equation (9) into equation (8) yields:

$$\log k/k_1 = (-E_a/2.303R)(1/T - 1/T_1)$$

$$= (-E_a/2.303R)[(T_1-T)/T_1T] \qquad (10)$$

(Lund, 1975).

Bigelow introduced the thermal death time (TDT) method. He observed that when the TDT, which is the minimum time to accomplish a total destruction, was plotted against temperature in °F, a straight line resulted. A typical TDT curve is shown in Figure 1.5. An equation describing the TDT curve is:

$$\log (TDT_1/TDT_2) = (-1/z)(T_1-T_2) = (T_2-T_1)/z \qquad (11)$$

where

TDT_1 and TDT_2 are the thermal death times at temperatures T_1 (min) and T_2 (min), respectively.

T_1 and T_2 are temperatures (°F) 1 and 2, respectively.

z is the °F temperature change required to change the TDT by a factor of 10.

The slope of the TDT curve, $-1/z$, characterizes the

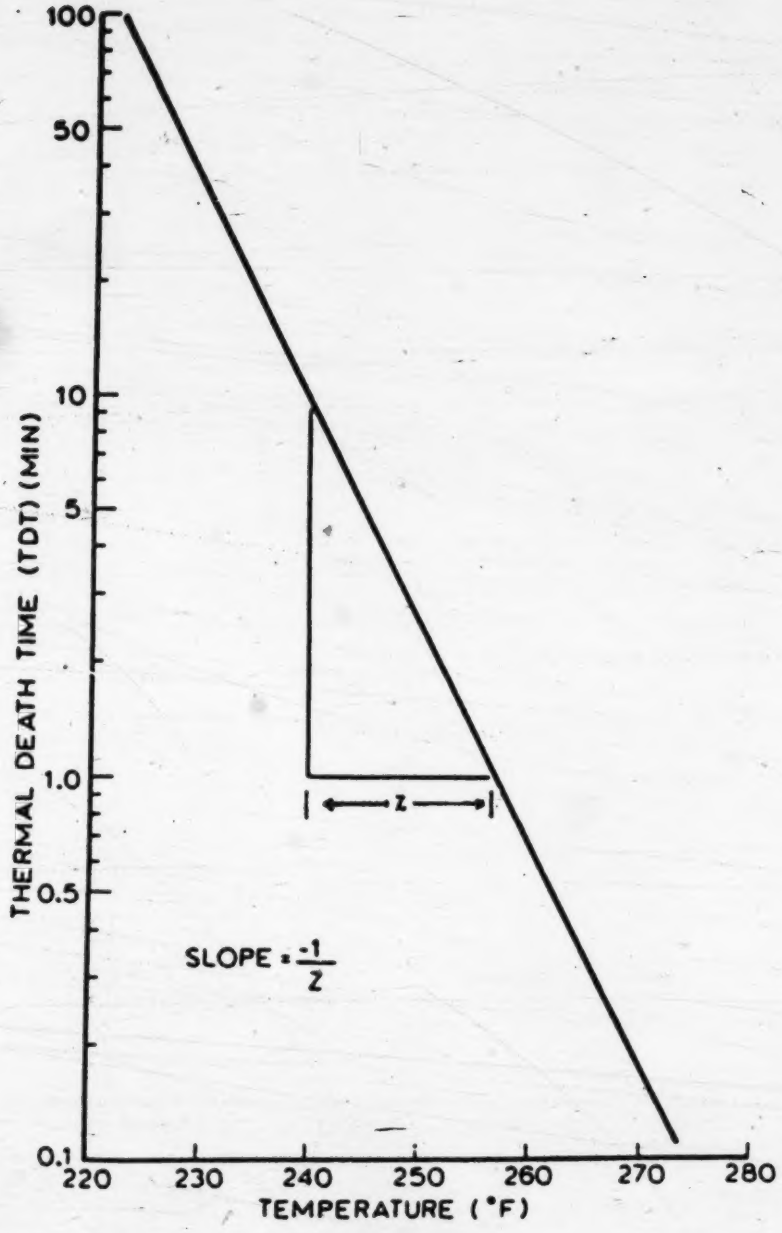


Figure 1.5 Thermal death time (TDT curve) for *Clostridium botulinum* in phosphate buffer. Source: Lund (1975).

dependence of the reaction rate constant on temperature and is therefore related to the activation energy. A small z value (10°F) indicates that the destruction time decreases by a factor of 10 for a 10 F° temperature increase, whereas a large z value (50°F) indicates the temperature must increase 50°F to reduce the destruction time by a factor of 10. Reactions that have small z values are highly temperature dependent, whereas reactions with large z values are not influenced much by temperature (Lund, 1975).

The symbol F represents the TDT for a microbial inactivation. The F value is dependent on temperature and is specific for one organism; it is identified with a superscript denoting the z value of the organism and a subscript denoting the temperature (°F). Substituting the F notation into equation (11) and using 250°F as a reference temperature yields:

$$\log (F_T^z/F_{250}^z) = (250-T)/z \qquad (12)$$

(Lund, 1975).

A similar equation can be developed for the decimal reduction value of a food component. This is referred to as a thermal resistance method and describes the effect temperature has on the time to reduce the concentration of the component by 90%. The thermal resistance equation is:

$$\log (D/D_1) = (T_1-T)/z \qquad (13)$$

Since k and D are related by equation (6), substitution into equation (13) results in:

$$\log (k_1/k) = (T_1-T)/z \quad or \quad \log (k/k_1) = (1/z)(T-T_1) \qquad (14)$$

(Lund, 1975).

Equation (14) states that the logarithm of the reaction rate constant is directly proportional to temperature. This contradicts equation (10), which states that the logarithm of the reaction rate is inversely proportional to the temperature. This concerned many investigators for many years, however it is recognized that over small temperature ranges $T \propto 1/T$, the systems (TDT and Arrhenius) are reconcilable (Lund, 1975).

The relationship between E_a and z can be obtained by equating equations (10) and (14) and changing °F in equation (14) to °K. Then:

$$(-E_a/2.303R)[(T_1-T)/T_1T] =$$

$$[(T-T_1)/z](5/9) = [-(T_1-T)/z](5/9) \qquad (15)$$

Rearranging yields:

$$E_a = (2.303RTT_1/z)(5/9) \qquad (16)$$

where

T_1 is the reference temperature (°K)

T is the temperature (°K)

1/z is the slope of the TDT curve (°F)

5/9 is the conversion factor of °F to °K

R is the gas constant (1.987 cal/mole-°K)

E_a is the activation energy (cal/mole)

(Lund, 1975).

1.3 Accelerated Shelf Life Testing

1.3.1 Scientific evaluation of shelf life

One of the major environmental factors that results in increased loss of quality and nutrition for most foods is exposure to increased temperature. The higher the temperature, the greater the loss of food quality (Labuza, 1982).

Food products are inherently unstable; their quality depends upon two factors: the length of time in storage and the storage temperature (Kwolek and Bookwalter, 1971). This is very important in research and development projects because the information obtained is required by those in the food industry so they can:

(1) evaluate the effect of the addition of ingredients or additives on shelf life,

(2) set an open date for the food on the package (e.g. a "best if used by" date) so that consumers are better informed in handling the product, and

(3) insure that the food meets the standards if nutritional labeling is used (that is, it does not fall below label value).

Information is required in order for the research scientist to make useful predictions about shelf life:

(1) the potential major modes for loss of quality of the product (Labuza, 1984),

(2) the factors which control the initial quality or

nutritional value at point of manufacture, including ingredients and process time, temperature, humidity, pH, etc.,

(3) the permeability of the package to various gases, such as O_2, CO_2 and water vapor, as well as light,

(4) the environmental conditions the food will be subjected to during distribution, including humidity, temperature and light, and

(5) the kinetics of the reactions leading to loss of quality and loss of nutritional value as a function of water activity, temperature, O_2 level, etc. (Labuza, 1979).

1.3.2 Temperature dependence on rate of deterioration

In order to predict the shelf life of a product without having to wait 12-24 months, accelerated shelf life testing (ASLT) procedures are used (Labuza and Schmidl, 1985).

To initiate shelf life testing, one must first make an educated guess as to the modes of deterioration which limit the shelf life of the product to be evaluated (Labuza and Schmidl, 1985).

The end of an acceptable shelf life is defined as that time stored samples are perceived as being "different" by a certain amount. In order to establish the rate of deterioration one must transform the data into a kinetic plot (Labuza and Schmidl, 1985).

To utilize the data, the Arrhenius model, or Q_{10} model, can be used to describe how much faster a reaction will go

if the product is held at some other temperature. If the temperature-accelerating factor is known, then extrapolation to lower temperatures, such as those found during distribution, could be used to predict expected product shelf life. The accelerating factor, or Q_{10} factor, is defined as:

$$Q_{10} = \frac{\text{rate at temperature } (T+10)}{\text{rate at temperature } T} \quad (17)$$

$$Q_{10} = \frac{\text{shelf life at temperature } T}{\text{shelf life at temperature } (T+10)}$$

$$= \theta s_{(T)} \; / \; \theta s_{(T+10)} \quad (18)$$

where

T is temperature in °C

θs is the shelf life at the indicated temperatures.

For any temperature difference, Δ, which is not 10 C°, this becomes:

$$Q_{10}^{\Delta/10} = \theta s_{(T1)} \; / \; \theta s_{(T2)} \quad (19)$$

(Labuza and Schmidl, 1985).

As previously discussed, the Arrhenius equation is as follows:

$$k = k_o \exp(-E_a/RT)$$

Utilizing the Arrhenius relationship as the basis to predict the rate of the reaction at any other temperature, one could evaluate the rate constant k at two or three different temperatures and then extrapolate with a straight line on a plot of ln k versus the reciprocal of the absolute temperature ($°K^{-1}$). If it is not possible to establish an Arrhenius plot, a plot of the log of time to end of shelf

life θs versus the temperature in C° can be used, as long as the extrapolation does not exceed a 30 C° range; this is called a shelf life plot. Thus, the value of either plot (ln k versus $°K^{-1}$ or log θs versus °C) is that data which can be obtained at higher temperatures and used to extrapolate to shelf life at some lower temperature. This is the principle behind ASLT (Labuza and Schmidl, 1985).

1.3.3 Errors associated with ASLT

There are a number of problems and theoretical errors associated with the ASLT conditions:

(1) Errors may occur in analytical or sensory evaluation (should be less than ±10%) (Labuza and Schmidl, 1985).

(2) As temperature increases, phase change may occur, e.g. solid fat going to liquid state, which can accelerate some reactions. The actual shelf life at the lower temperature may then become shorter than predicted.

(3) Carbohydrates in the amorphous state may crystallize out at higher temperatures, creating more free water for other reactions. The error in prediction of shelf life will be incorrect.

(4) Control samples are usually frozen prior to storage, thus reactants become concentrated in the unfrozen liquid. Storage below the thaw point will lead to prediction errors.

(5) If two reactions with different Q_{10} values can cause a quality loss in a food at higher temperature, the one with the higher Q_{10} may predominate at higher temperatures but via a different mechanism. Hence, the low Q_{10} reaction may

predominate at normal storage conditions, thus causing prediction errors. For example, lipid oxidation and losses of fat-soluble vitamins (A and E) in dehydrated potatoes predominate below 31°C and browning and lysine losses predominate above this temperature.

(6) When temperature increases, the water activity of dry foods can also increase. Therefore, there will be an increase in reaction rate for products of low water activity in sealed packages.

(7) For each 10°C increase in temperature, the solubility of gases, especially oxygen, in fat or water decreases by almost 25%. An oxidative reaction (loss of vitamins E, A or C) can decrease in rate if oxygen is a limiting factor. At higher temperatures the rate will be lower than the theoretical rate and thus result in an underprediction of true shelf life at normal storage temperatures.

(8) The product should be placed in a totally impermeable pouch when stored in high-temperature, low-humidity cabinets so as to avoid moisture loss since this will lead to shorter predicted shelf life at the lower temperature.

(9) At sufficiently high temperatures proteins may become denatured and this can cause erroneous prediction of shelf life.

(10) Many reactions, such as non-enzymatic browning, are pH-dependent. The pH of the system is a function of temperature. Neutrality shifts from pH 7.00 at 25°C to pH 7.47 at 0°C (Labuza and Riboh, 1982).

In the real world of distribution, products undergo temperature fluctuations and shelf life will actually be shorter than at constant room temperature (Labuza and Schmidl, 1985).

Q_{10} values range from 1.5-2 for sensory quality loss in canned foods, 1.5-3 for rancidity, 4-10 for browning reactions and 20-40 for quality loss of some fruits and vegetables (Labuza, 1984).

1.3.4 Open dating benefits versus nonbenefits

The National Canners' Association (NCA), which represents the canning industry as a whole, has suggested a system of voluntary open dating for canned foods which would be uniform throughout the United States (Labuza, 1982).

According to the NCA, the date of the pack would be the easiest to implement, but unfortunately would not tell the consumer anything about the shelf life of the product. This would create a problem with canners who have seasonal packs because the date of the cans would seem old when actually the product is still within its shelf life. The canning industry assumes that a three-year shelf life for most fruits and vegetables is the norm, and a one-year delay would still result in a more than adequate shelf life for a given product. The consumer would have to be convinced of this fact and this would require much time, effort and money. There is also possibility for confusion among consumers who would mistake the "pack date" for a "use by date" (Labuza, 1982).

The "sell by date" is not really applicable to canned foods, which are often stored in the home for a long period of time after being sold. If some system which indicates shelf life beyond the selling date were adopted, it could be the best system (Labuza, 1982).

The "best if used by" date could be the most useful to consumers because it would give an idea of the shelf life of the product if conditions of storage were known or uniform. This would also be beneficial at the grocery level for rotation of stock. Furthermore, a "date of best quality if used by" could be printed on the can with or without an explanation of the temperature of storage on which it is based. Ideally, the canning industry could collect data on each product for each mode of deterioration, estimate time-temperature distributions and then estimate shelf life at several home storage conditions. If this is not done, then at least a "pack date" would be useful (Labuza, 1982).

1.4 Research Objectives

The objective of this study was to examine the stability of 5'-purine ribonucleotides at various pH's to identify the hydrolysis rates of these compounds during canning conditions.

There is very little literature on the stability of these nucleotides. Hashida *et al.* (1968) observed a 52-61% recovery of flavor nucleotides in canned seafood and Hashida *et al.* (1966) found about a 74% recovery in canned mushrooms. Canning of corned beef, retorted 30 min at 120°C,

resulted in 94% retention of IMP and GMP, and 60 min at 120°C resulted in 78% retention of the two nucleotides (Ajinomoto, 1982c). Nguyen (1984) reported that, when IMP and GMP were hydrolysed for 30 min at 124°C, 65.9% and 41.3%, respectively, were degraded to their corresponding nucleosides.

In view of the lack of stability of these nucleotides under canning conditions, the final objective was to use ASLT to establish the stability of the three ribonucleotides at room temperature.

2. EXPERIMENTAL

2.1 General

HPLC grade water was prepared using a Millipore Milli-Q system (Millipore Corp., Bedford, MA).

Potassium dihydrogen phosphate, HPLC grade was obtained from Fisher Scientific (Fairlawn, NJ). All other solvents and reagents were analytical grade or better.

Standards were obtained as follows: adenosine 5'-monophosphate, adenosine, adenine, guanosine 5'-monophosphate disodium salt and guanine from Aldrich Chem. Co. (Milwaukee, WI); inosine and guanosine from Terochem (Edmonton, Alta.); inosine 5'-monophosphate disodium salt and hypoxanthine from Sigma Chem. Co. (St. Louis, MO).

Stock solutions were prepared at a concentration of 12.575 mg IMP or GMP per ml H_2O. A Gilson pipetman (Gilson Medical Electronics, France) was calibrated with water prior to measuring aliquots of 0.2353 ml stock solution into 100 ml volumetric flasks. The flasks were made up to volume with 0.02 M phosphate buffer. This gave a final concentration of 30 µg IMP or GMP per ml buffer. Aliquots of 3,000 µg AMP were dissolved directly into the phosphate buffer and transferred to 100 ml volumetric flasks and made up to volume with buffer. Hydrolysis at various pH's was studied, including pH 3, 4, 4.5, 5, 5.5, 6, 6.5, 7 and 8.

2.1.1 Hydrolysis

Aliquots of 0.5 ml samples (30 µg/ml) were transferred into Pyrex tubes (6 mm o.d.). Tubes (7 cm) were sealed at one end, filled, and sealed at the other end using an oxygen-gas flame.

The entire set of Pyrex tubes was submerged in a high temperature silicone oil bath. A magnetic stirrer stirred the oil and an Ika-Werk Staufen (Germany) contact thermometer maintained the bath at 121°C. A Fluke 2240B datalogger (John Fluke Mfg. Co., Inc., Mountlake Terrace, WA) fitted with five thermocouples placed at various points throughout the bath was used to ensure the temperature was 121°C. Each 15 min up to 3 hr, tubes were removed, rapidly submerged in an ice bath and then placed in the freezer for later analysis.

2.1.2 Accelerated shelf life testing

Tubes containing 30 µg nucleotide/ml were prepared as above in 0.02 M phosphate buffer at pH 5. The hydrolysis was carried out in the oil bath for 6 hr at 106°C, 23 hr at 91°C, 53 hr at 76°C and 88 days at 53°C. At recorded intervals, tubes were removed from the oil bath, rapidly submerged in an ice bath and placed in a freezer for later analysis.

2.2 HPLC Analysis

2.2.1 Analysis of hydrolysis

The Pyrex tubes were defrosted, opened and 100 µl aliquots were analyzed using high performance liquid chromatography (HPLC). The HPLC system consisted of a Beckman model 110A pump (Beckman Instr. Co., Palo Alto, CA) fitted with a Rheodyne model 7125 injector (Terochem, Edmonton, Alta.). The Whatman (Whatman, NJ) analytical columns used were the Rapid Analysis column (RAC II) strong anion exchange (SAX), 10 cm x 4.6 mm i.d., and strong cation exchange (SCX), 25 cm x 4.6 mm i.d., which were protected by a 7 cm x 2.1 mm i.d. guard column containing the same pellicular ion-exchanger as the analytical column. A pre-injector 25 cm x 4.6 mm i.d. precolumn containing silica gel was attached. The mobile phase consisted of phosphate buffer which was degassed prior to use. A Laboratory Data Control Spectro-Monitor II Ultra Violet (UV) detector, model 1240A (Milton Roy, Riviera Beach, FL) was used for the detection of nucleic acid compounds with a wavelength set at 254 nm. A Hewlett Packard model 3388A Integrator Terminal (Hewlett Packard, Edmonton, Alta) was attached to the UV detector.

Nucleotides and the decomposition compounds from nucleotides were analyzed using SAX and SCX columns, respectively. The concentration, pH and flow rate of buffer in the HPLC mobile phase were as follows (Nguyen, 1984):
0.017M, pH 4.00, flow rate 1.0 ml/min for analysis of

nucleotides.

0.01M, pH 3.60, flow rate 1.0 ml/min for analysis of nucleosides and bases.

2.2.2 Analysis of accelerated shelf life testing

The Pyrex tubes were defrosted, opened and 60 µl aliquots were analyzed using HPLC. A Guardian pump model 300LC (Scientific Systems, Inc., State College, PA) was fitted with a Rheodyne model 7125 injector (Terochem, Edmonton, Alta.) and was attached to the RAC II SAX column, 10 cm x 4.6 mm i.d., which was protected by a 7 cm x 2.1 mm i.d. guard column. The mobile phase consisted of phosphate buffer; it was degassed prior to use. An LDC/Milton Roy Spectro Monitor D variable wavelength detector (LDC/Milton Roy, Riviera Beach, FL) was used for the detection of the nucleotide with the wavelength set at 254 nm. A Varian 4270 Integrator (Varian Assoc. Inc., Walnut Creek, USA) was attached to the UV detector. 0.017 M phosphate buffer at pH 4.00 was the HPLC mobile phase. The flow rate for the analysis of nucleotides was 1.0 ml/min.

2.3 Analysis of Data

If the concentration of the nucleotide is represented by c, the first-order rate law can be written as:

$$-dc/dt = kc \qquad (20)$$

If the initial concentration, at time t=0, is c_0, and, if at some later time t the concentration has fallen to c, the integration gives:

$$-\int_{c_0}^{c} dc/c = k \int_0^t dt \qquad (21)$$

and

$$-\ln c/c_0 = \ln c_0/c = kt \qquad (22)$$

A reaction is said to be a first-order reaction if a plot of $\ln c_0/c$ versus t is a straight line. The slope of the line can be used to obtain the value of the rate constant k (Barrow, 1981). A plot of the rate constant k versus pH gives the rate profile for the respective nucleotide at a given temperature.

For a first-order reaction it is customary to use not only the rate constant, k, for the reaction but also the related quantity, the half-life of the reaction. The half-life is defined as the time required for the concentration of the nucleotide to decrease to half its initial value. For a first-order reaction the relationship of the half-life, $t_{1/2}$, to the rate constant can be found from equation (22) by substituting at $t=t_{1/2}$ the concentration $c=\frac{1}{2}c_0$. Therefore, one obtains (Barrow, 1981):

$$\ln c_0/\tfrac{1}{2}c_0 = kt_{1/2} \qquad (23)$$

$$t_{1/2} = 0.693/k \qquad (24)$$

In order to calculate how much time is required to achieve the half-life of the individual nucleotide at room temperature, accelerated shelf life testing was performed. As previously stated, both the Arrhenius plot and the shelf life plot could be utilized. In the first case, a plot of ln k as a function of the reciprocal of absolute temperature (1/T) gives a straight line with the slope of the line being the activation energy divided by the gas constant. In the

second case, a plot of time to the end of shelf life (assumed to be $t_{1/2}$) on semi-log paper versus the temperature in °C can be composed. In both cases the line could be extrapolated to shelf life at some lower temperature, such as room temperature.

3. RESULTS AND DISCUSSION

3.1 Hydrolysis of 5'-Purine Ribonucleotides

Acid hydrolysis of nucleosides (inosine, guanosine and adenosine) or nucleotides (IMP, GMP, AMP) generally releases the purine bases (hypoxanthine, guanine and adenine), while neutral or alkaline hydrolysis of nucleotides liberates only the phosphate residues (Harbers *et al.*, 1968). The rate of degradation depends on both the heating time and sample pH.

The SAX column was used to determine the undegraded nucleotides after hydrolysis. Measurement of peak areas of different concentrations gave a linear response for all three nucleotides.

The pH-rate profiles for IMP, GMP and AMP are shown in Figures 3.1, 3.2 and 3.3, respectively. Tables 3.1, 3.2 and 3.3 present the data for all three nucleotides, respectively. The aqueous hydrolysis of the nucleotides at the various pH's is expressed as a first-order reaction rate. The SCX column was used to determine the nucleosides and purine bases which are the breakdown products of nucleotides. Samples at or near the half-life at each pH were examined for nucleoside and purine base amounts. It was determined that at the half-lives of the nucleotides two mechanisms were occurring at pH 3 with both nucleoside and purine base in large amounts, indicating that both phosphate and base hydrolysis were occurring. Significant amounts of purine base were not revealed between pH 4-8, suggesting that hydrolysis occurred mainly through phosphate cleavage.

Table 3.1 Hydrolysis data for inosine 5'-monophosphate (IMP) at 121°C and various pH's.

pH	Rate constant, k (min^{-1})	Standard error of estimate	Coefficient of correlation
3	2.141×10^{-2}	5.0×10^{-4}	0.988
4	1.111×10^{-2}	2.4×10^{-4}	0.990
4.5	1.092×10^{-2}	4.3×10^{-4}	0.992
5	1.103×10^{-2}	4.1×10^{-4}	0.994
5.5	1.036×10^{-2}	3.2×10^{-4}	0.995
6	9.682×10^{-3}	3.7×10^{-4}	0.993
6.5	6.681×10^{-3}	2.0×10^{-4}	0.996
7	5.545×10^{-3}	6.8×10^{-4}	0.938
8	1.855×10^{-3}	6.9×10^{-5}	0.974

Table 3.2 Hydrolysis data for guanosine 5'-monophosphate (GMP) at 121°C and various pH's.

pH	Rate constant, k (min^{-1})	Standard error of estimate	Coefficient of correlation
3	2.202×10^{-2}	5.0×10^{-4}	0.990
4	1.510×10^{-2}	4.6×10^{-4}	0.982
4.5	1.686×10^{-2}	2.7×10^{-4}	0.995
5	1.692×10^{-2}	2.7×10^{-4}	0.996
5.5	1.578×10^{-2}	3.3×10^{-4}	0.992
6	1.534×10^{-2}	3.4×10^{-4}	0.990
6.5	1.110×10^{-2}	2.0×10^{-4}	0.992
7	6.749×10^{-3}	1.3×10^{-4}	0.992
8	2.881×10^{-3}	8.9×10^{-5}	0.981

Table 3.3 Hydrolysis data for adenosine 5'-monophosphate (AMP) at 121°C and various pH's.

pH	Rate constant, k (min^{-1})	Standard error of estimate	Coefficient of correlation
3	1.531×10^{-2}	4.0×10^{-4}	0.987
4	1.198×10^{-2}	2.5×10^{-4}	0.991
4.5	1.212×10^{-2}	4.0×10^{-4}	0.981
5	1.360×10^{-2}	4.9×10^{-4}	0.977
5.5	1.057×10^{-2}	3.0×10^{-4}	0.986
6	1.000×10^{-2}	2.6×10^{-4}	0.988
6.5	7.858×10^{-3}	2.3×10^{-4}	0.985
7	4.865×10^{-3}	1.5×10^{-4}	0.983
8	2.679×10^{-3}	2.8×10^{-4}	0.894

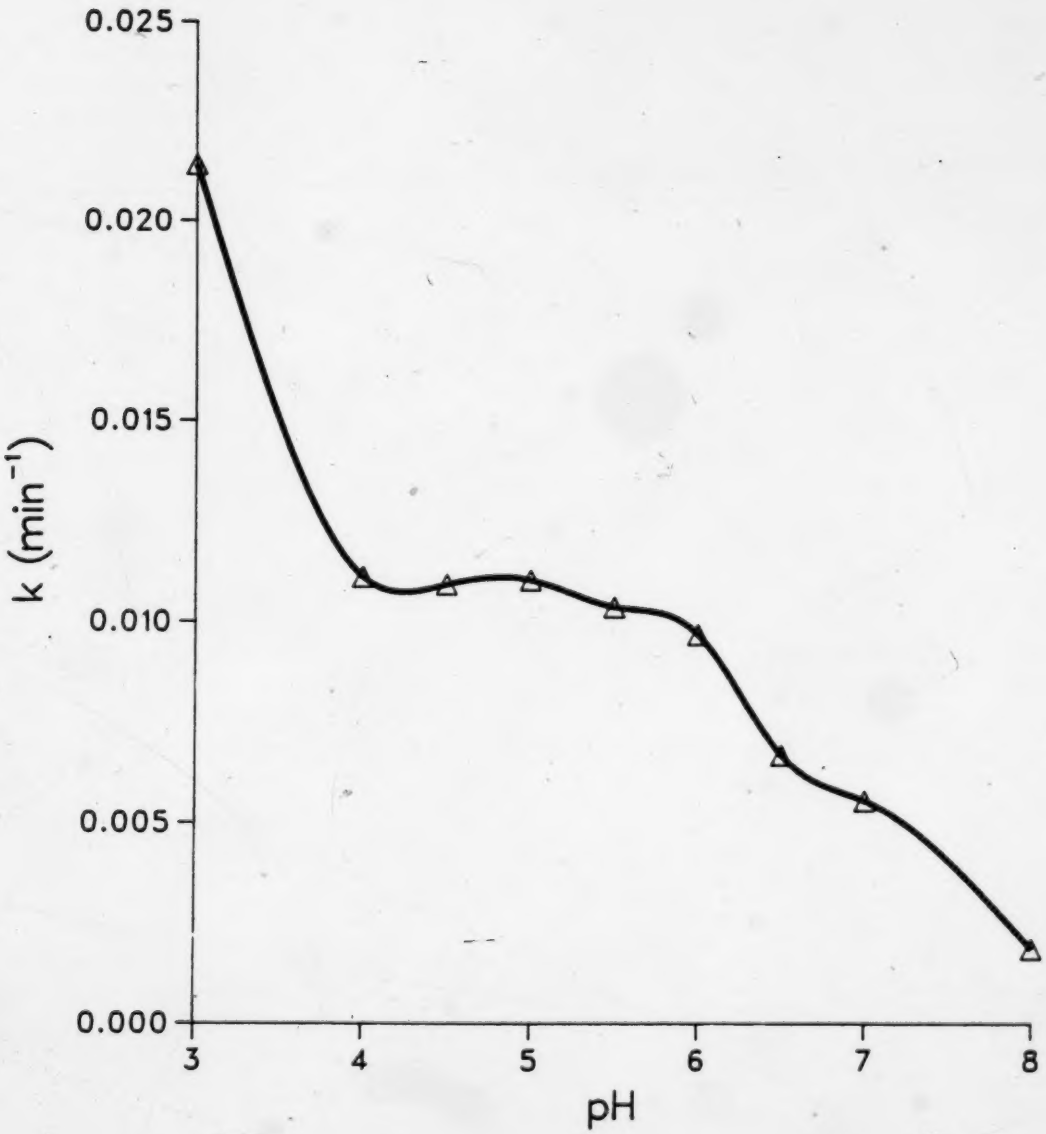


Figure 3.1 pH-Rate profile for inosine 5'-monophosphate (IMP) at 121°C.

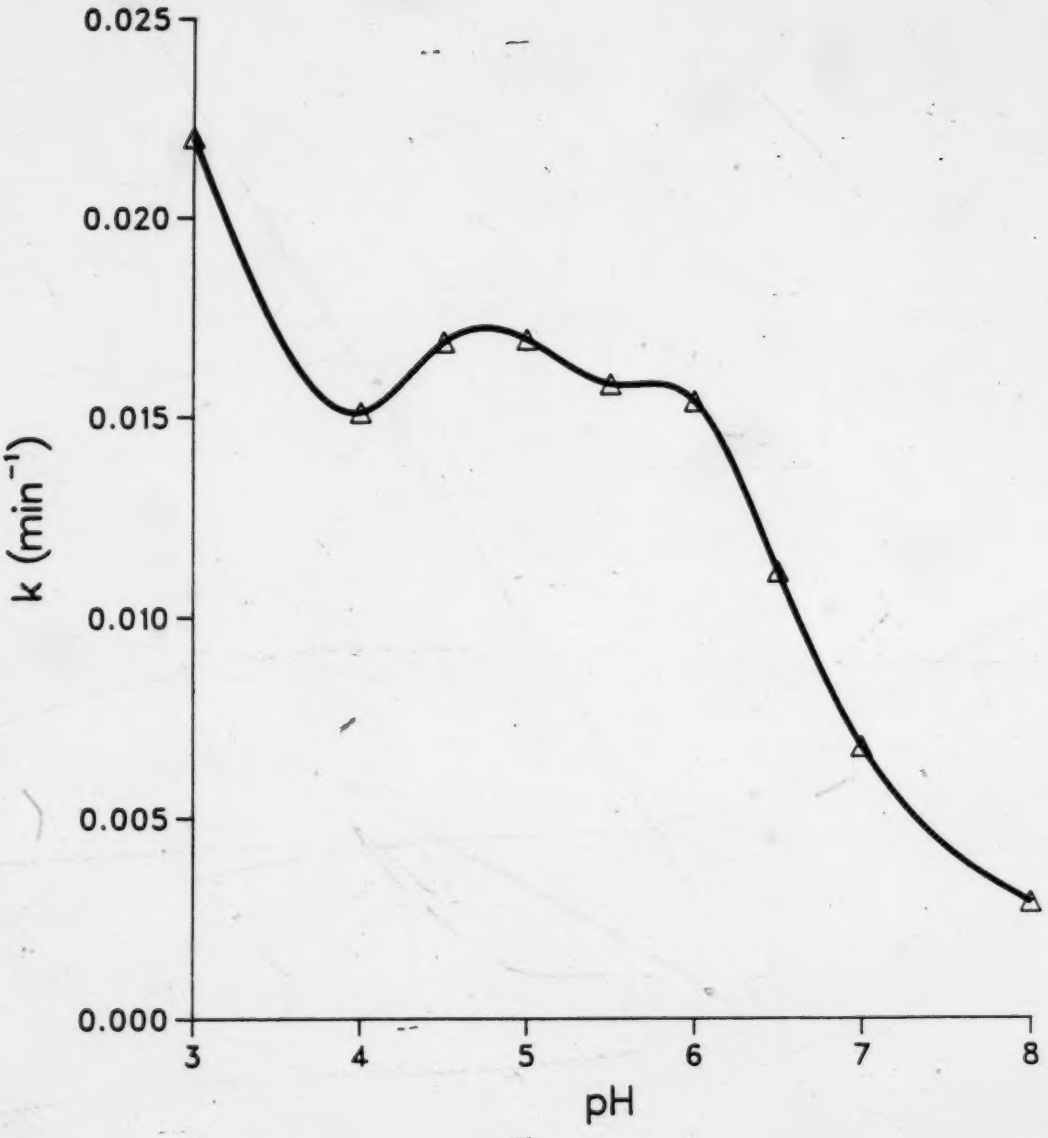


Figure 3.2 pH-Rate profile for guanosine 5'-monophosphate (GMP) at 121°C.

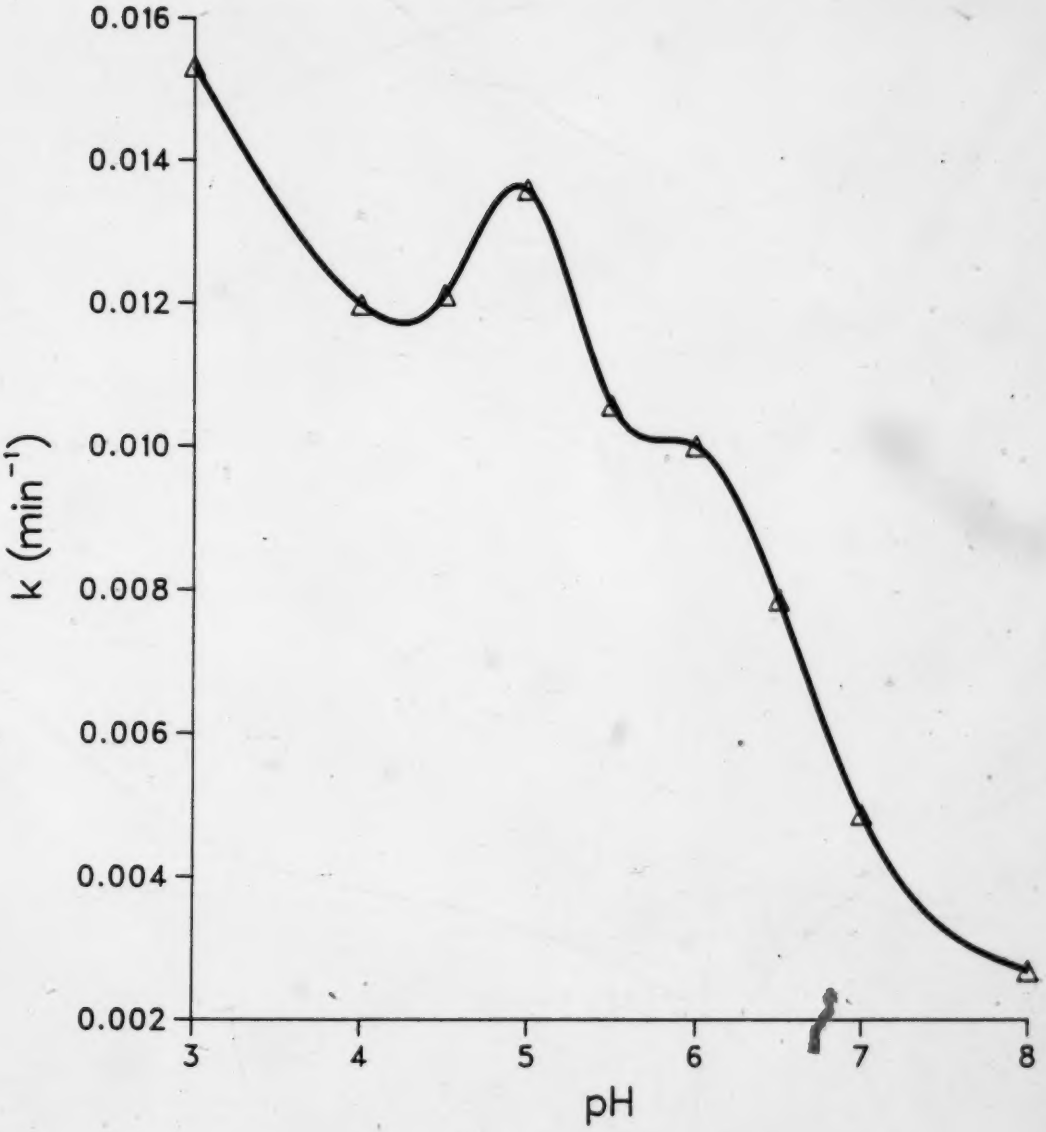


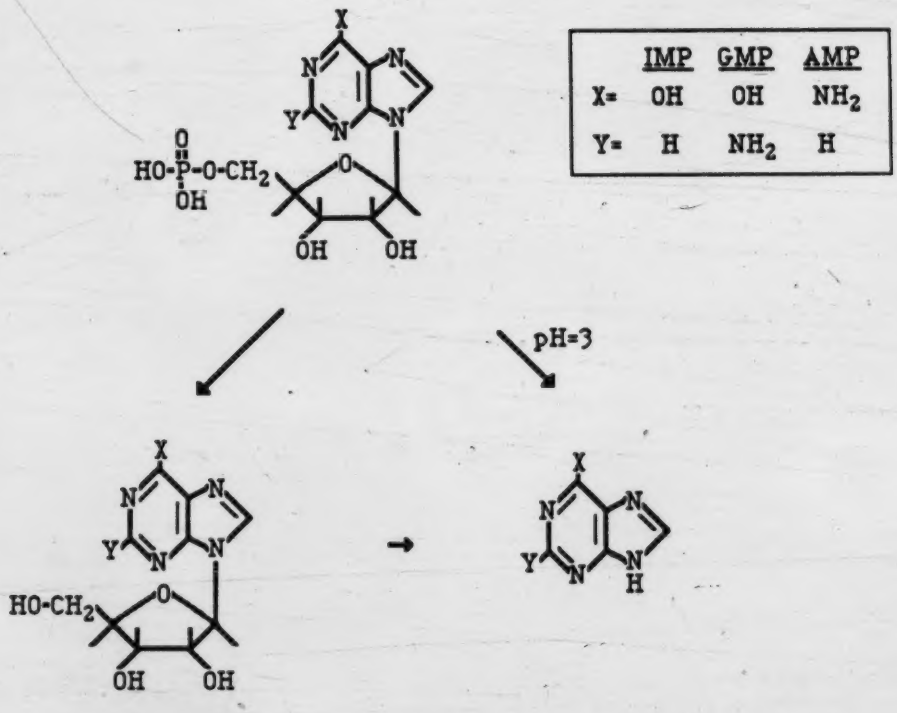
Figure 3.3 pH-Rate profile for adenosine 5'-monophosphate (AMP) at 121°C.

With only very small amounts of purine base hydrolysis detected for the nucleotides at pH's of 4 or higher and since purine nucleoside base hydrolysis rates are greater under these conditions (Nguyen, 1984), the curves in Figures 3.1, 3.2 and 3.3 can be explained as follows. Hydrolysis of the purine nucleotides at pH's higher than 4 occurred initially by phosphate hydrolysis to the nucleoside. That is, this was the rate determining step. Small amounts of purine base hydrolysis could then occur from the more unstable nucleoside produced. More purine base hydrolysis from the nucleoside occurred at the more acidic pH's in this range because of acid catalysis of this reaction (Figure 3.4). At pH 3.0, however, the acid strength was sufficient to result in purine base hydrolysis directly from the nucleotide. The operation of both purine base and phosphate hydrolysis of the nucleotides at this pH resulted in the increased hydrolysis rates detected.

Phosphate and purine base hydrolyses will be analyzed separately in sections 3.1.1 and 3.1.2, respectively.

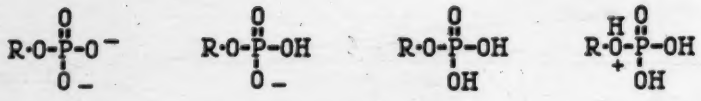
3.1.1 Phosphate hydrolysis of nucleotides

In a monosubstituted ester of phosphoric acid there are four molecular species, differing in degree of protonation, which may undergo reaction. They are the dianion (1), monoanion (2), neutral (3) and conjugate acid (4) species (Barnard *et al.*, 1955).



	IMP	GMP	AMP
X=	OH	OH	NH_2
Y=	H	NH_2	H

Figure 3.4 Degradation of 5'-purine nucleotides.

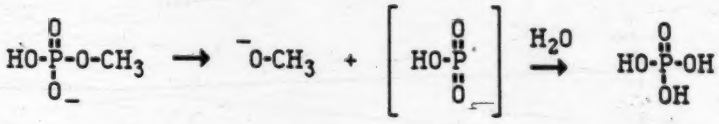


(1) (2) (3) (4)

Many authors have shown that the best conditions for hydrolysis of monophosphate esters occur between pH 4-5, where the monoanion has the maximum concentration. Also, the hydrolysis of these monophosphate esters follows first-order kinetics.

Barnard *et al.* (1955) investigated the hydrolysis of methyl phosphate. They revealed that on either side of pH 4.16 the rate of hydrolysis decreases. Isotope ^{18}O was used to show the involvement of phosphorus-oxygen bond fission. They suggested that the mechanism of the hydrolysis of the monoanion can be formulated in two ways. First, the water molecule attacks the phosphorus atom,

or, second, a heterolysis of the phosphorus-oxygen bond may take place, giving the hypothetical metaphosphoric acid which rapidly hydrates to give orthophosphoric acid:



At the time, the exact nature of the mechanism was not known, but was found to be common to simple monosubstituted phosphates (Barnard *et al.*, 1955).

In 1958, the pH-rate profile for monophosphate esters was confirmed by Bunton *et al.*. The rate of hydrolysis was very small in alkaline solution, increased to a maximum at pH 4, then fell to a minimum at about pH 0.5 and rose again in even more strongly acidic solutions. It was postulated that the maximum rate of hydrolysis was about pH 4 and was due to the reaction of the monoanion (Figure 3.5). At pH 4.17, 99.9% of the monomethyl phosphate was present as the monoanion. It was suggested that the monoanion is more reactive than either the dianion or the neutral species since the negative charge of the O^- group is imagined as the "driving force" of the reaction (Bunton *et al.*, 1958). The dianion $ROPO_3^=$ does not react readily with hydroxide ion because of the electrostatic repulsion of the mutual negative charges on the ions. This helps explain why the rate is greater at pH 4 than in alkaline solutions (Butcher and Westheimer, 1954).

In 1954, Butcher and Westheimer proposed that the hydrolysis of monoesters of phosphoric acid at pH 4 proceeds by way of an intermediate (5):

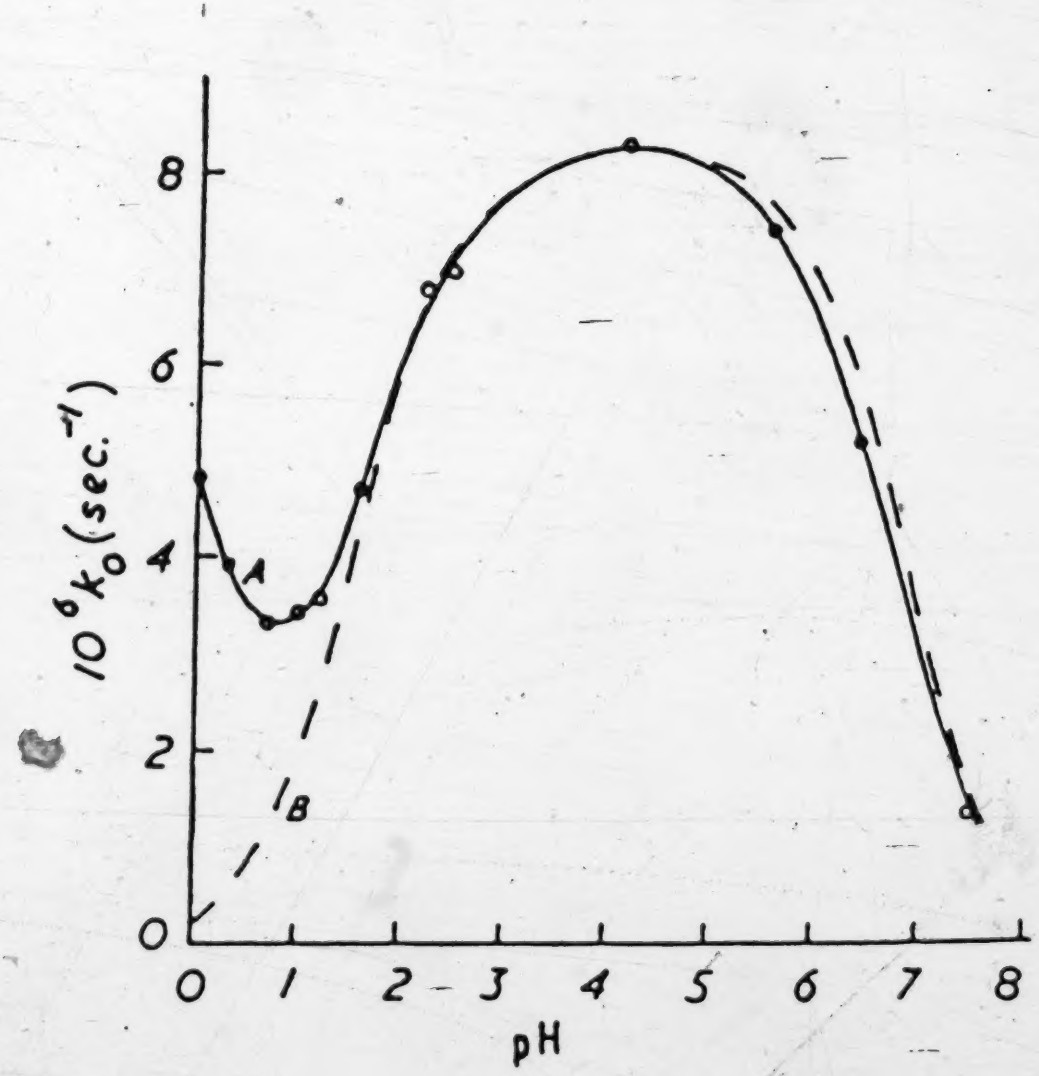
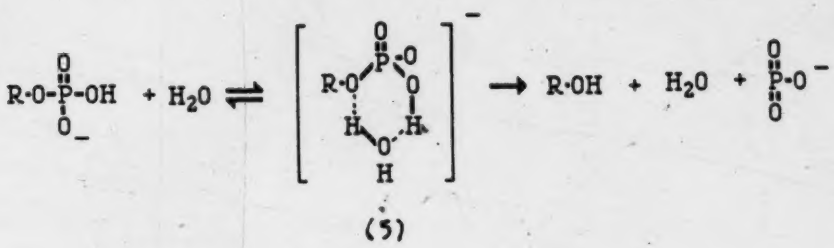
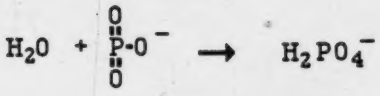


Figure 3.5 Hydrolysis of monophosphate ester at 100°C (Bunton *et al.*, 1958). A. experimental; B. calculated.



(5)



The alcohol and the monomeric metaphosphate ion, PO_3^-, are postulated as primary products of the hydrolysis. Monomeric metaphosphates have been postulated as intermediates in phosphorylations (Butcher and Westheimer, 1954).

A plot of hydrolysis reaction rate against pH for potassium dihydrogen phosphate showed a maximum at pH 5 (Bunton *et al.*, 1961), hydrolysis of acetyl phosphate indicated that the maxiumum was at pH 4.7 (Disabato and Jencks, 1961a,b) and hydrolysis of ethyl phosphate passes through a maximum between pH 4-5 (Cox and Ramsay, 1964). In all cases, the rate maximum corresponded to the monoanion and the hydrolysis followed first-order reaction kinetics. The bond between phosphorus and oxygen has been broken exclusively in the reaction of the monoanion; this has been established in a sufficient number of cases to indicate generality of the result. Variations in structure produce slight variations in the pH-rate profile maximum.

In 1979, Lee studied the kinetics of inosine 5'-monophosphate at 100°C in a pH range of 5.52-6.87. The rate was confirmed to be a first-order reaction and a rate-pH profile

showed a maximum near the pK_a (6.1) of 5'-IMP. The rate of 5'-IMP was not influenced by ionic strength. The product of 5'-IMP heat decomposition was determined to be inosine with trace amounts of hypoxanthine (Lee, 1979).

Presentation of the ionization constants for the nucleotides (Figures 3.6 and 3.7; Hirokawa *et al.* (1985) and Ts'o (1974)) demonstrates that the maximum concentration of the monoanion (two species) would occur in the pH range of 1.0-6.4. The maximum rate of phosphate hydrolysis of the three nucleotides (Figures 3.1-3.3) in the pH range of 4-8 occurred at pH 5. This would suggest that phosphate hydrolysis took place at a greater rate from the monoanion phosphate species with the uncharged purine base, although the mechanism of phosphate hydrolysis in this pH range is complex, as shown by the complexity of the curves. Figure 3.8 illustrates the phosphate hydrolysis of these 5'-purine nucleotides.

3.1.2 Purine base hydrolysis of nucleosides

Nucleosides, fragments of ribonucleic acid (RNA) containing a purine or pyrimidine base and a ribose group, may be hydrolysed to their base and sugar components by acid-catalysed reactions (Zoltewicz *et al.*, 1970).

Acid-catalysed hydrolysis of purine nucleosides is generally accepted to occur via the mechanism originally proposed by Shapiro (Figure 3.9). The acid-catalysed hydrolysis takes place by the reaction of mono- and diprotonated forms (Zoltewicz and Clark, 1972). Protonation

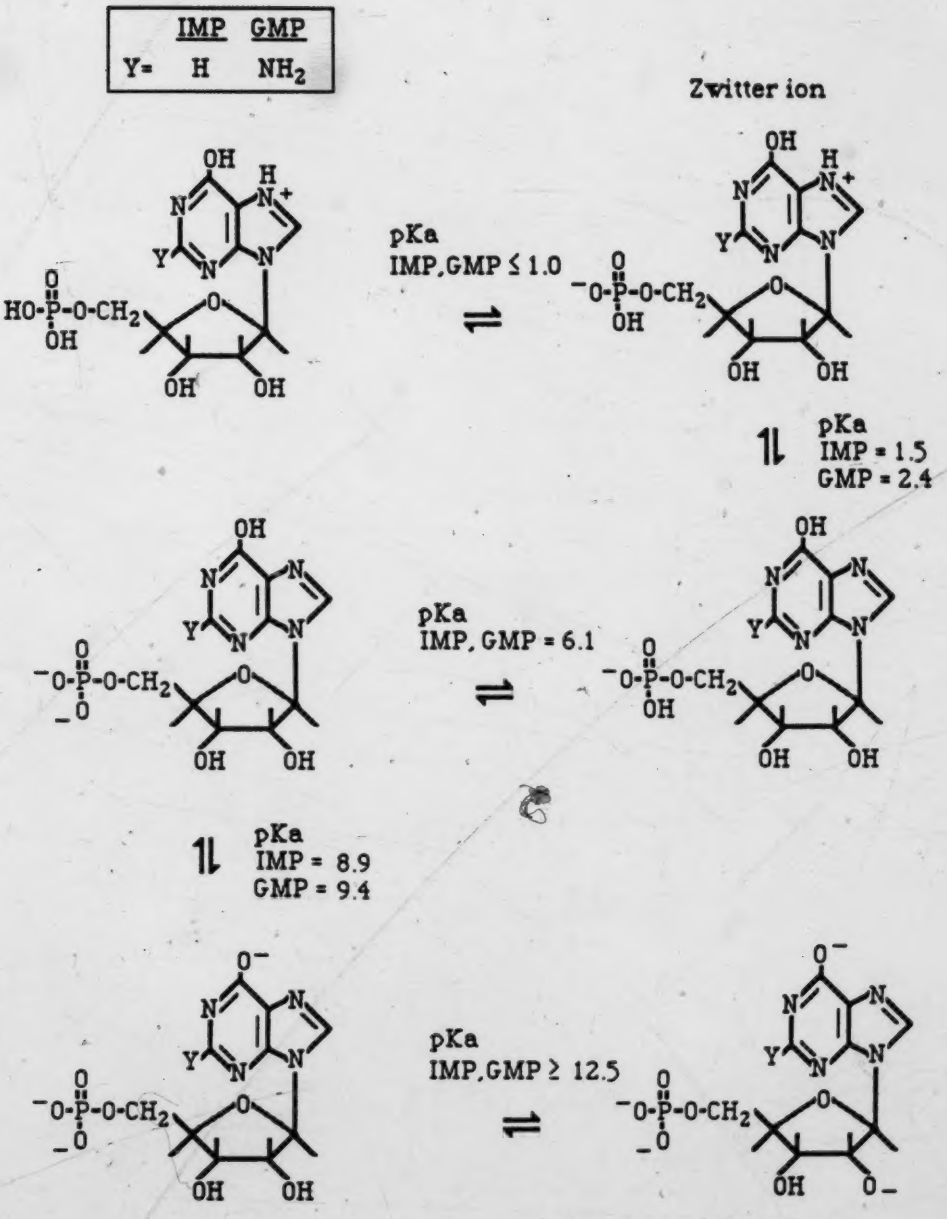


Figure 3.6 Ionization constants of inosine 5'-monophosphate (IMP) and guanosine 5'-monophosphate (GMP).

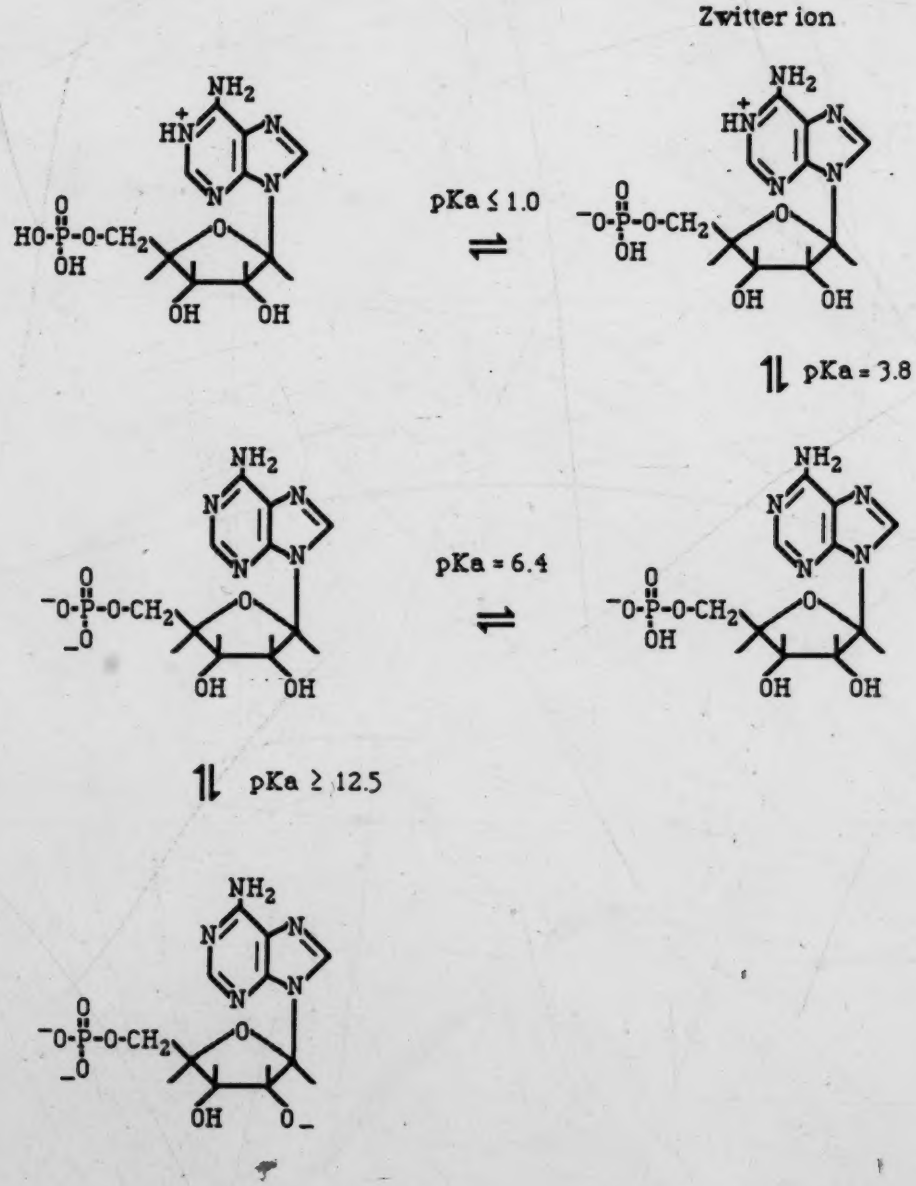
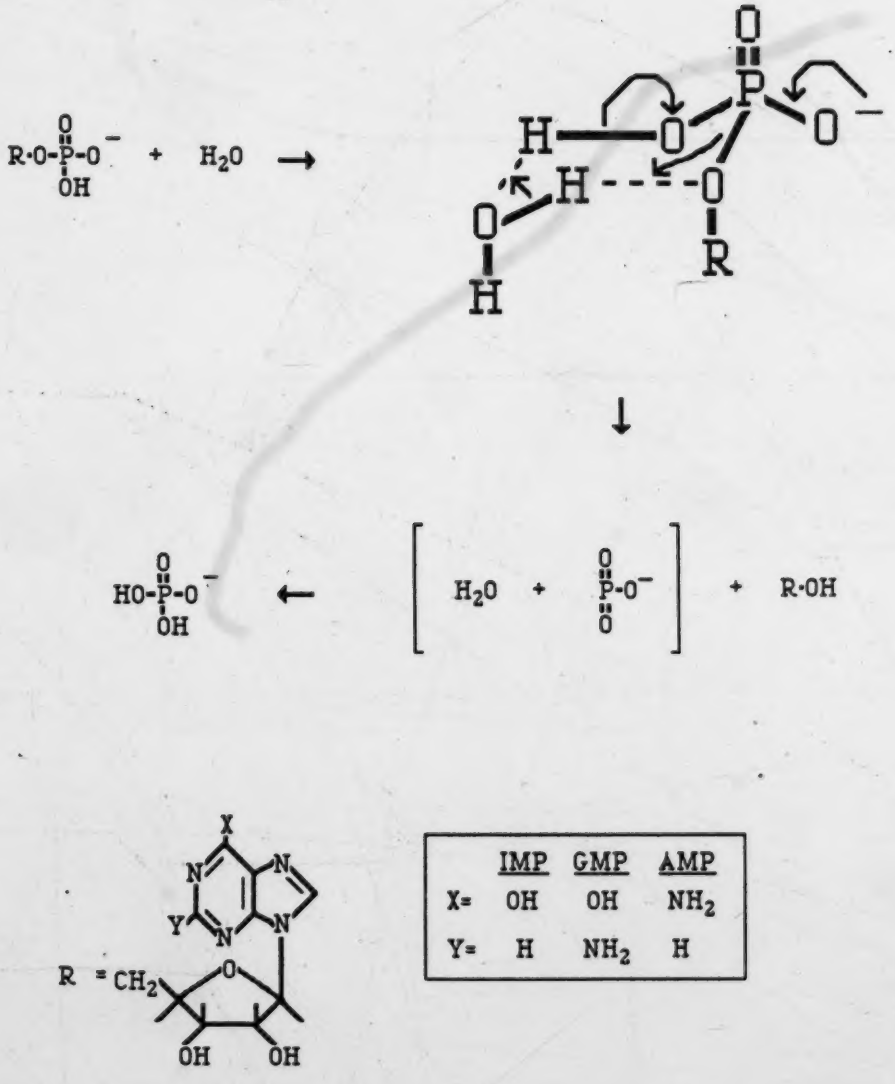


Figure 3.7 Ionization constants of adenosine 5'-monophosphate (AMP).



	IMP	GMP	AMP
X=	OH	OH	NH_2
Y=	H	NH_2	H

Figure 3.8 Phosphate hydrolysis of 5'-purine nucleotides.

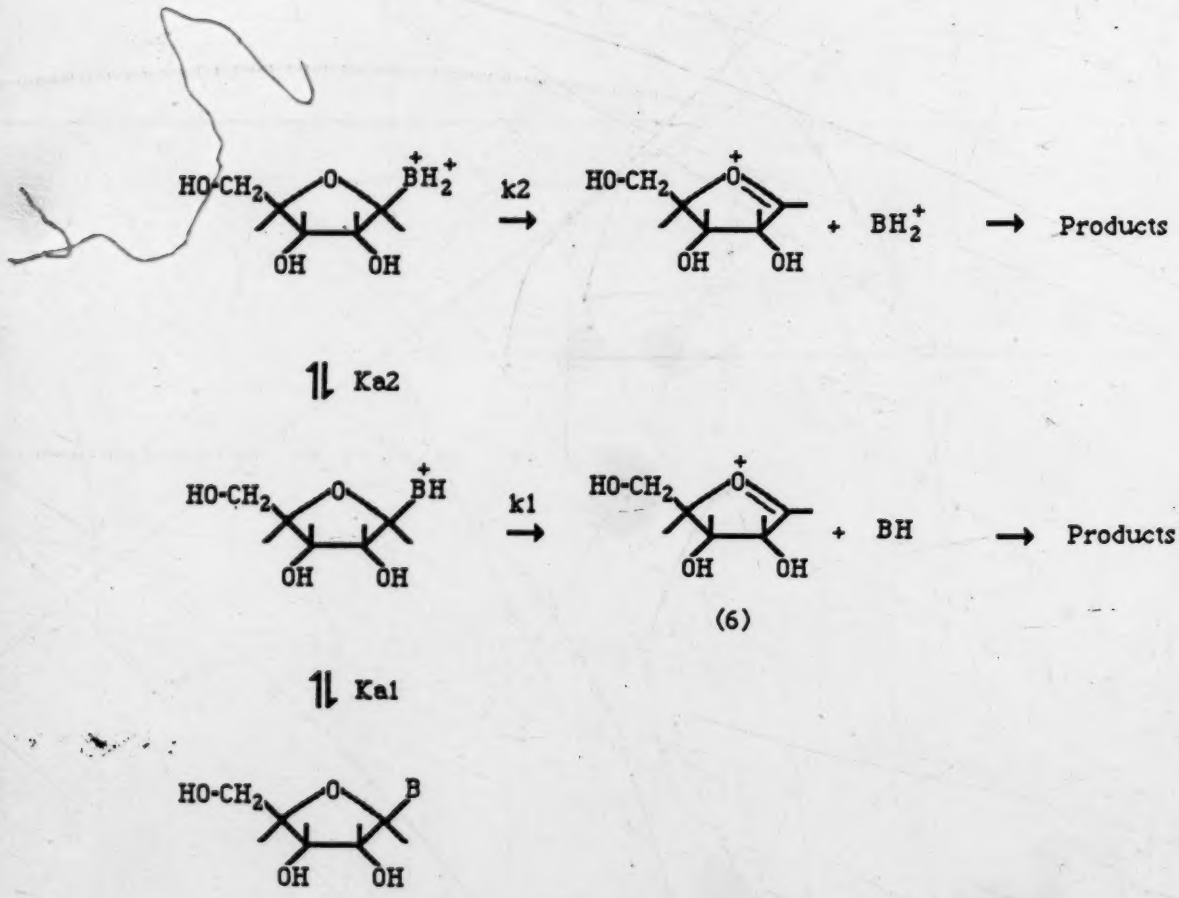


Figure 3.9 Shapiro's nucleoside hydrolysis (Hatfield, 1984).

of the purine moiety occurs, followed by purine-glycosyl bond cleavage, giving rise to an oxocarbonium ion intermediate (6) which then reacts with solvent (Stein *et al.*, 1978; Zoltewicz *et al.*, 1970).

The concentration of diprotonated species is negligible at pH 3, therefore acid hydrolysis at this pH would occur via the monoprotonated species.

It is well established that electronegative groups in the sugar moiety stabilize purines to acid-catalysed hydrolysis (Smith *et al.*, 1961; Sporns, 1977). Phosphorylation of the sugar moiety of nucleotides destabilizes the oxocarbonium ion intermediate formation and thus retards the sugar-base cleavage (Robins and Basom, 1973). Golankiewicz *et al.* (1985) reported that 5'-monophosphates are usually more stable than their respective nucleosides. Others have shown that the presence of a phosphate group is responsible for the rate retardation (Shapiro and Danzig, 1973). The acid hydrolysis of 2-deoxynucleosides proceeds faster than their ribo counterparts (Garrett and Mehta, 1972) since the 2'-hydroxyl group increases the electron-withdrawing effect, destabilizing the oxocarbonium ion intermediate (Zoltewicz *et al.*, 1970).

Panzica *et al.* (1972) suggested that the site of protonation might be expected to influence the rate of hydrolysis of purine nucleosides. Protonation of purine nucleosides takes place on the purine moiety. Specific sites of protonation on the purine moiety differ for the various nucleosides, for example, adenosine and guanosine protonate

at N-1 and N-7, respectively (Panzica *et al.*, 1972).

The only pH where significant purine base hydrolysis was observed was pH 3.0 or the more acidic pH, as expected. The electron-withdrawing effect of the 5'-phosphate group stabilized the nucleotides to purine base hydrolysis at higher pH's. The small amount of purine base hydrolysis at these higher pH's could be explained as occurring from the more acid-labile nucleosides.

3.2 Accelerated Shelf Life Testing

Accelerated shelf life testing (ASLT) was performed to establish when half of the nucleotide concentration of the respective nucleotides (that is, the half-life of the nucleotide) was lost. The ASLT data for the individual nucleotides (IMP, GMP and AMP) are given in Tables 3.4, 3.5 and 3.6.

The Arrhenius plot (Figure 3.10), which is a graph of ln k versus the reciprocal of the absolute temperature, reveals straight lines for all three nucleotides. Employing the equation for a straight line,

$$y = mx + b \qquad (25)$$

and the data in Table 3.7 (which were determined from the Arrhenius plot), the half-life of each nucleotide could be evaluated from the rate constant at room temperature (extrapolation of Arrhenius plot data). The half-lives of IMP, GMP and AMP were determined to be 35.68, 18.65 and 39.66 years. This clearly indicates that nucleotides are very stable at room temperature.

Table 3.4 ASLT data for inosine 5'-monophosphate (IMP) at pH 5 and various temperatures.

T°C	Rate constant, k (h^{-1})	Standard error of estimate	Coefficient of correlation
121	6.618×10^{-1}	2.7×10^{-4}	0.994
106	1.580×10^{-1}	5.3×10^{-3}	0.988
91	3.391×10^{-2}	1.1×10^{-3}	0.995
76	5.157×10^{-3}	2.9×10^{-4}	0.959
53	2.322×10^{-4}	2.3×10^{-5}	0.924

Table 3.5 ASLT data for guanosine 5'-monophosphate (GMP) at pH 5 and various temperatures.

T°C	Rate constant, k (h^{-1})	Standard error of estimate	Coefficient of correlation
121	1.015×10^{-0}	2.7×10^{-4}	0.996
106	2.276×10^{-1}	2.9×10^{-3}	0.998
91	5.159×10^{-2}	1.4×10^{-3}	0.996
76	8.948×10^{-3}	4.9×10^{-4}	0.969
53	3.979×10^{-4}	2.7×10^{-5}	0.963

Table 3.6 ASLT data for adenosine 5'-monophosphate (AMP) at pH 5 and various temperatures.

T°C	Rate constant, k (h^{-1})	Standard error of estimate	Coefficient of correlation
121	8.160×10^{-1}	4.0×10^{-4}	0.977
106	1.164×10^{-1}	5.5×10^{-3}	0.975
91	3.483×10^{-2}	1.9×10^{-3}	0.981
76	4.685×10^{-3}	3.6×10^{-4}	0.968
53	2.313×10^{-4}	1.6×10^{-5}	0.958

Table 3.7 ASLT data for determining the shelf life of the three nucleotides by the Arrhenius method.

Nucleotide	Slope (°K)	Standard error of estimate of slope	y-intercept (h^{-1})	Coefficient of correlation
IMP	-1.509×10^4	1.9×10^2	3.798×10^1	0.999
GMP	-1.478×10^4	1.8×10^2	3.757×10^1	0.999
AMP	-1.521×10^4	4.9×10^2	3.826×10^1	0.998

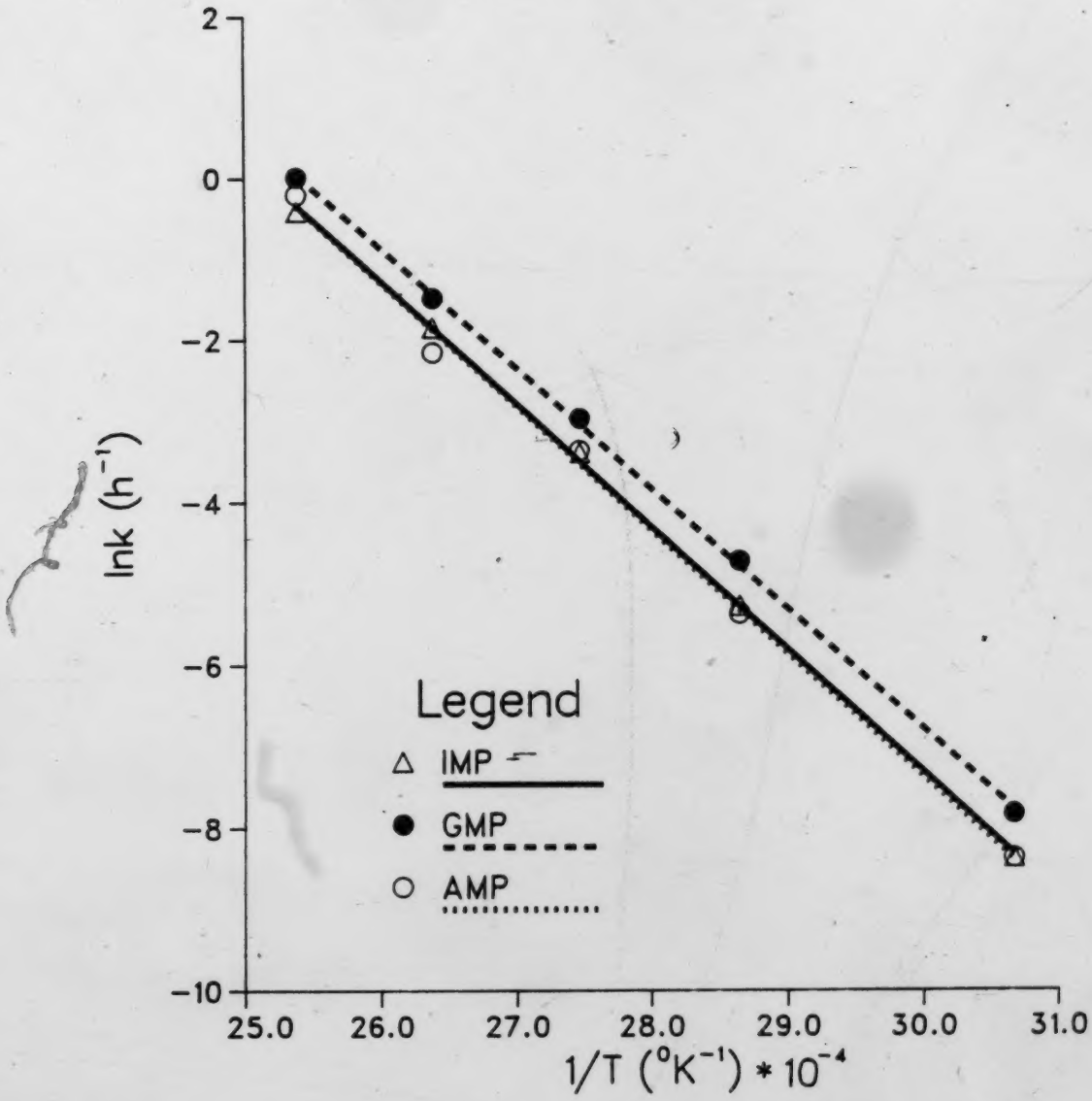


Figure 3.10 Arrhenius plot of the nucleotides

Out of interest, an alternative method for determining shelf life was utilized. A shelf life plot (Figure 3.11), which is a graph of the log of the half-life versus the temperature in degrees Celcius, was established by allowing the time to the end of shelf life (θs) to be the half-life. The half-lives were evaluated by using the straight line equation (Eq'n 25) and the data in Table 3.8 (which were determined from the shelf life plot). The half-lives of IMP, GMP and AMP at 23°C were 3.99, 4.83 and 9.99 years.

The two methods are not comparable for several reasons:

(1) Labuza and Schmidl (1985) stated that the shelf life plot should only be employed if an Arrhenius plot is not possible to establish.

(2) In a small temperature range (20-40°C change) the Arrhenius plot can be approximated by the shelf life plot (Labuza, 1982). In this case, a 70°C change was investigated, thus only the Arrhenius plot should be used.

(3) The shelf life plot could be employed as long as the extrapolation does not exceed a 30°C range. For this study, this range was pushed to the limits (extrapolation from last experimental point at 53°C to 23°C), leading to erroneous results.

(4) Figure 3.12 compares the extrapolation of the shelf life plot at 43, 33 and 23°C to that of the Arrhenius plot for AMP. The Arrhenius plot gives a straight line and extrapolation leads to a divergence with the shelf life plot.

Table 3.8 ASLT data for determining the shelf life of the three nucleotides at RT by the shelf life method.

Nucleotide	Slope ($°C^{-1}$)	Standard error of estimate of slope	y-intercept (h)	Coefficient of correlation
IMP	-5.09×10^{-2}	2.3×10^{-3}	6.067×10^{0}	0.997
GMP	-4.99×10^{-2}	2.2×10^{-3}	5.773×10^{0}	0.997
AMP	-5.14×10^{-2}	2.2×10^{-3}	6.124×10^{0}	0.997

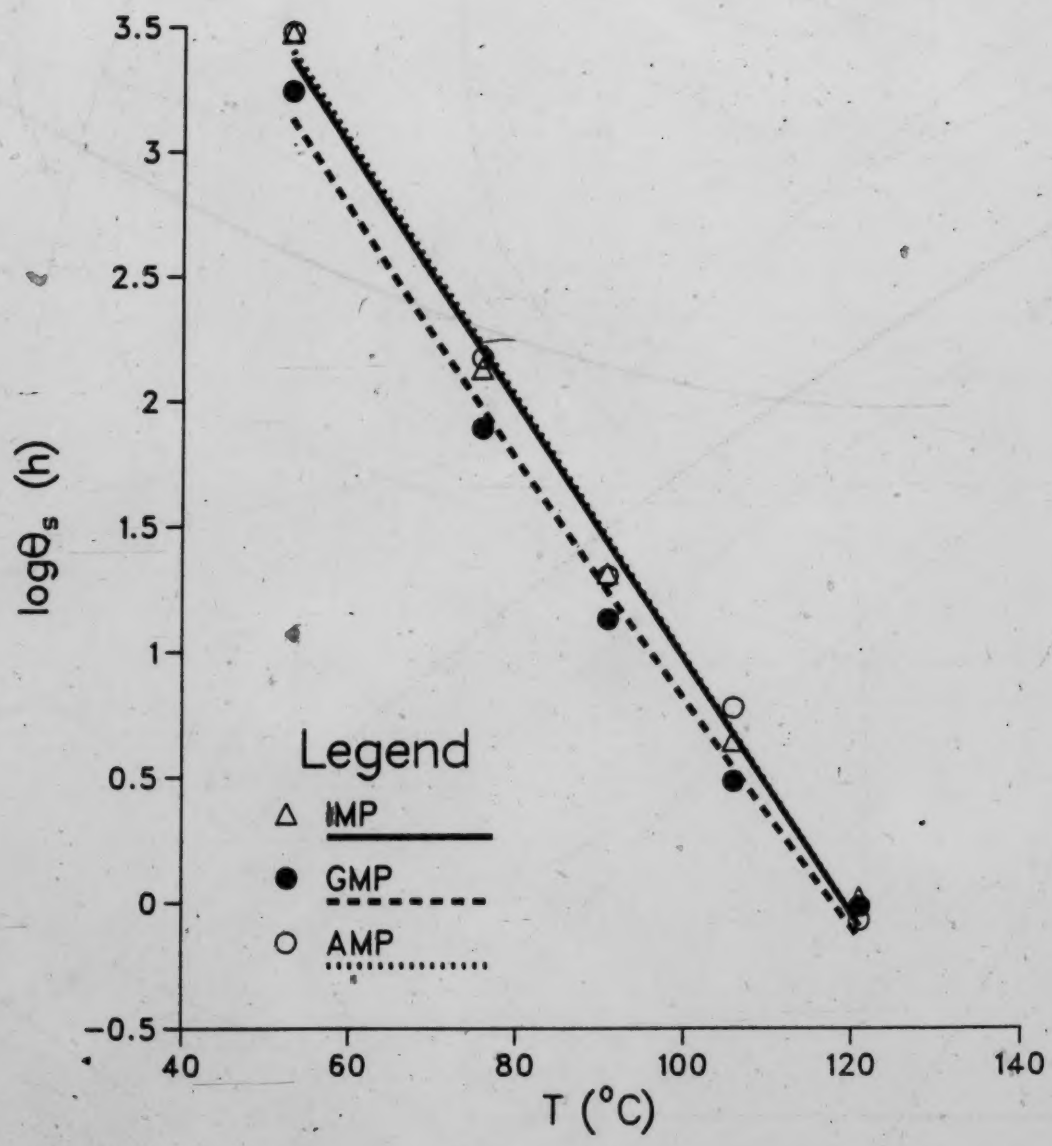


Figure 3.11 Shelf life plot of the nucleotides.

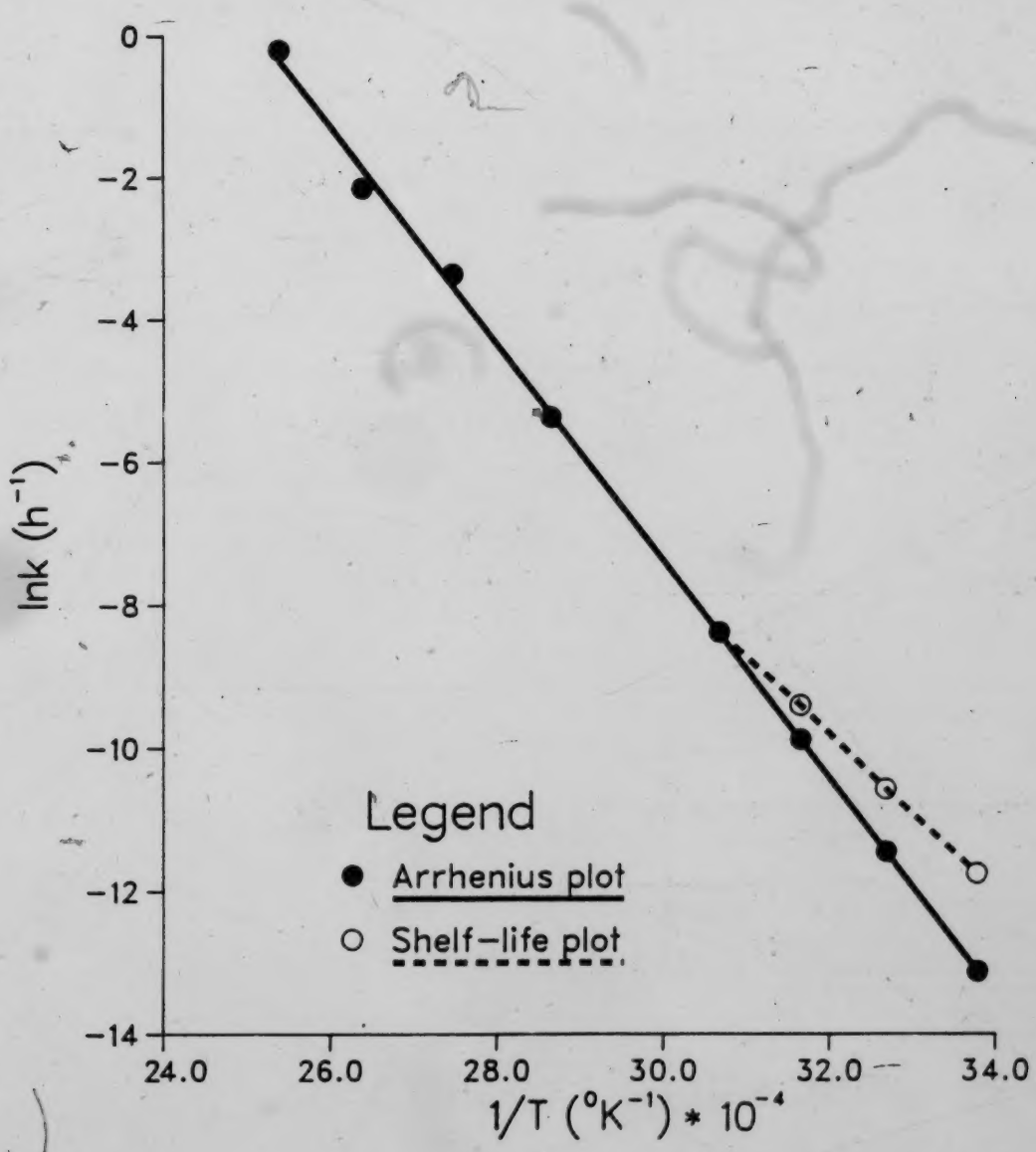


Figure 3.12 Comparison of the extrapolation of the shelf life plot to the Arrhenius plot for AMP.

To conclude, the shelf lives of all three nucleotides were very stable at room temperature. The Arrhenius method was found to be a more exact method for determining shelf life, whereas the shelf life method was found to be inappropriate for this work.

3.3 Suggestions for Future Research

Most of the ASLT in food science is presently carried out employing the shelf life method. This method, which underestimates shelf life, is based on empirical relationships rather than well established chemical kinetics such as the Arrhenius method. In view of the vast differences in estimation of shelf life using the two methods, a detailed study should be carried out on the reliability of the shelf life method.

Such a study could involve the estimation of shelf life utilizing both ASLT methods for a known accurately measurable defect such as microbial or chemical spoilage in food. This investigation would be followed by an actual measurement of the shelf life at the extrapolated conditions.

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